An external ecological niche for *Candida albicans* within reducing, oxygen-limited zones of wetlands and river banks

by
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Thesis presented in partial fulfilment of the requirements for the degree Master of Science at the University of Stellenbosch

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March 2011

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Summary

The ascomycetous yeast, *Candida albicans*, has been almost exclusively studied in a clinical context, due to the medical risk and costs associated with the yeast. Most environmental research into the external survival of this opportunistic pathogen has been concerned with short-term, severe pollution challenges. However, a study of literature indicates that the habitat characteristics of the oxygen-limited zones in wetlands and riverbanks are comparable to those of the gastrointestinal source of sewage-borne *C. albicans*. Interestingly, these are the external, environmental regions to which sewage-borne *C. albicans* is often exposed. In addition, oxygen-limitation is the predominant parameter in stimulating conjugation of *C. albicans*. Based on these observations, this study aimed to assess polluted river bank and wetland environments in the Western Cape of South Africa as potential habitats to accommodate a niche for *C. albicans*, particularly comparing the presence of this yeast in oxygen-limited, plant debris-rich zones and aerobic, clear, flowing zones. The second objective was to employ *in vitro* microcosm studies to investigate the survival and growth of *C. albicans* in various microhabitats similar to those comprising the oxygen-limited zones of wetlands. These included the rhizosphere of wetland flora, various soil and mud types and decomposing plant debris. The final objective was to establish the presence of sufficient nutrient and energy sources within this environment for the growth of *C. albicans*. In particular, cellulosic substrates and mono- and disaccharides released by the natural degradation of wetland plant debris were investigated as potential energy sources for this human commensal in the wetland environment. These study objectives combined to demonstrate the potential of such an oxygen-limited, plant debris-rich environment as a niche for *C. albicans* external to its human host.

Both semi-quantitative culturing techniques and quantitative Real-Time PCR demonstrated the improved survival of *C. albicans* in oxygen-limited, plant debris-rich zones in wetland and river bank environments, in comparison to aerobic, clear subsurface water zones in the same environments. These zones were compared in the Plankenburg and Diep Rivers, situated in the Western Cape of South Africa. Correlations between coliform concentrations and total yeast concentrations were demonstrated in each of the different river zones, with higher pollution levels characteristic of the dry season. *Candida albicans* numbers in flowing water (zone W), rock-filtered (zone R) and plant-filtered water (zone P) were compared during the progress of the rainy and dry seasons. No *C. albicans* was observed in clear, flowing water throughout the analysis. Early in the rainy season, both rock-filtered (aerobic, poor in plant debris) and plant-filtered (oxygen-limited, rich in plant debris) water demonstrated

C. albicans numbers at approximately equivalent levels of 10^2-10^3 cells/100 mL. However, as the rainy season progressed and total yeast and coliform numbers in all zones of the rivers dropped to negligible levels, *C. albicans* could no longer be detected in aerobic, rock-filtered zones; but its numbers remained at constant levels in oxygen-limited, plant-filtered zones. This suggests that oxygen-limited wetland and river bank zones rich in plant matter, analogous to the human gastrointestinal tract, may provide an ideal habitat in which *C. albicans* could establish a niche external to its host.

The survival of this yeast in the various microhabitats that comprise this anaerobic, reducing wetland environment was evaluated with *in vitro* microcosms. The rhizosphere of wetland plants had no influence on *C. albicans* growth and survival in comparison to bulk soil away from the plant, and wetland mud microbiota was demonstrated to be inhibitory to its survival. However, decaying plant debris was shown to increase the survival of the yeast in this inhibitory mud environment. *Candida albicans* was shown to compete well saprophytically in anaerobic plant debris microcosms. In addition, the tendency of *C. albicans* to associate with plant matter in an aquatic environment was demonstrated by inoculating the yeast in water containing *Hydrilla*, a submerged macrophyte found in South African aquatic environments. Plate and liquid analyses, as well as an ANKOM NDF analysis, indicated unequivocally that the *C. albicans* strains evaluated in this work were unable to utilise the complex carbohydrates of the wetland habitat, including cellulose and fibre. However, HPLC, along with GC-MS, demonstrated the anaerobic assimilation by *C. albicans* of monosaccharides released by natural lignocellulose degradation of wetland plant matter. An analysis of total nitrogen by digestion in a nitrogen analyser, as well as evaluation of ammonium, nitrate and nitrite in a KCL extract, also showed that *C. albicans* assimilates nitrogenous compounds released by the decomposition of wetland plant matter. This decay process occurs constantly in wetland and river bank habitats. It may therefore provide energy and nutrients for *C. albicans*, particularly in the anaerobic zones where conjugation may possibly occur and a niche may be established, as indicated by the results obtained for the Plankenburg and Diep Rivers.

Samevatting

Die askomisete gis *Candida albicans* is feitlik eksklusief in ‘n kliniese konteks bestudeer weens die mediese risiko en koste daaraan verbonde. Die meeste omgewingsnavorsing op die eksterne oorlewing van hierdie opportunistiese patogeen was toegespits op die uitdagings van ernstige korttermyn besoedeling. ‘n Literatuurstudie toon egter dat die habitat-eienskappe van die suurstof-beperkte sones in vleilande en rivieroewers vergelykbaar is met dié van die gastroïntestinale bron van *C. albicans* wat in riool gevind word. Interessant genoeg is dit juis hierdie eksterne omgewingsgebiede waaraan *C. albicans* vanuit riool dikwels blootgestel word. Hierby is suurstof-beperking die vernaamste parameter in die stimulering van konjugasie in *C. albicans*. Op grond van hierdie waarnemings poog dié studie om besoedelde vleilande en rivieroewers in die Wes-Kaap Provinsie van Suid-Afrika te evalueer as potensiële habitatte wat ‘n nis van *C. albicans* kan akkommodeer, en veral om die teenwoordigheid van hierdie gis in suurstof-beperkte sones ryk aan plantafval te vergelyk met aerobe, helder, vloeiende sones. Die tweede doelwit was om *in vitro* mikrokosmos studies te gebruik om die oorlewing en groei van *C. albicans* in verskillende mikrohabitatte soortgelyk aan suurstof-beperkte sones in vleilande te ondersoek. Dit sluit die risosfeer van vleilandflora in, asook verskillende grond- en moddertipes en ontbindende plantafval. Die laaste doelwit was om die teenwoordigheid van genoegsame voedings- en energiebronne in dié omgewing te bepaal vir die groei van *C. albicans*. In besonder is sellulose substrate, asook die mono- en di-sakkariede, wat deur die natuurlike afbraak van vleiland plantafval vrygestel word, as potensiële energiebronne van hierdie mens-kommensaal in die vleiland-omgewing ondersoek. Hierdie studiedoelwitte het gesamentlik die potensiaal van so ‘n suurstofbeperkte, plantafvalryke omgewing as ‘n nis vir *C. albicans* buite die menslike gasheer aangetoon.

Beide semi-kwantitatiewe kweektegnieke en kwantitatiewe in-tyd PKR het die verbeterde oorlewing van *C. albicans* in suurstofbeperkte, plantafvalryke sones in vleiland en rivieroeweromgewings gedemonstreer, in teenstelling met aerobe, helder oppervlakwatersones in dieselfde omgewings. Hierdie sones in die Plankenburg- and Dieprivier in die Wes-Kaap Provinsie, Suid-Afrika, is met mekaar vergelyk. Korrelasies tussen coliform konsentrasies en totale giskonsentrasies is in elk van die verskillende sones in dié riviere gedemonstreer, met hoër vlakke van besoedeling kenmerkend aan die droër seisoen. *Candida albicans* getalle in vloeiende water (sone W), rots-gefiltreerde (sone R) en plant-gefiltreerde water (sone P) is deur die verloop van die reën- en droë seisoene met mekaar vergelyk. Geen *C. albicans* is deur die loop van die analises in helder, vloeiende water bespeur nie. Vroeg in die reënseisoen het beide rots-gefiltreerde (aerobe, min plantafval) en plant-gefiltreerde

(suurstofbeperk, ryk in plantafval) water vergelykbare vlakke van *C. albicans* getoon, naamlik 10^2-10^3 selle/100 mL. Soos die reënseisoen egter verloop het en die totale gis- en coliforme getalle in al die sones van die riviere tot weglaatbare vlakke gedaal het, kon *C. albicans* egter nie meer in die aerobe, rots-gefiltreerde sones bespeur word nie, hoewel die getalle in suurstofbeperkte, plant-gefiltreerde sones konstant gebly het. Dit dui daarop dat suurstof-beperkte vleiland en rivieroewer sones ryk in plantmateriaal, analoog tot die menslike gastroïntestinale kanaal, die idealke habitat mag bied waarin *C. albicans* 'n nis mag vind buite sy gasheer.

Die oorlewing van hierdie gis in die verskillende mikrohabitatte wat uit hierdie anaerobe, reduserende vleilandomgewing bestaan, is met *in vitro* mikrokosmosse geëvalueer. Die risosfeer van vleilandplante het in vergelyking met die grond weg van die plant geen effek op die groei en oorlewing van *C. albicans* gehad nie, en vleiland modder-mikrobiota is gevind om die oorlewing daarvan te inhibeer. Verrottende plantafval het egter die oorlewingsvlakke van giste in hierdie inhiberende modderomgewing verbeter. *Candida albicans* kan egter saprofities goed kompeteer in anaerobe plantafval mikrokosmosse. Hierby is die geneigdheid van *C. albicans* om met plantmateriaal in waterige omgewings te assosieer gedemonstreer deur die gis te innokuleer in water wat *Hydrilla*, 'n onderwater makrofiet wat in Suid-Afrikaanse akwatiese omgewings aangetref word, bevat. Plaat en vloeibare analises, asook 'n ANKOM NDF data-analise, het onteenseglik getoon dat die *C. albicans* stamme wat in dié werk gebruik is, nie in staat was om die komplekse koolhidrate, insluitende sellulose en vesel, van die vleiland habitat te benut nie. HPLC, saam met GC-MS, toon egter *C. albicans* se anaerobe assimilasie van monosakkariede wat deur natuurlike lignosellulose afbraak van vleiland plantmateriaal vrygestel is. 'n Totale stikstof analise deur vertering in 'n stikstof analiseerder, en 'n evalueering van ammonium, nitraat en nitriet in 'n KCl ekstrak, het ook getoon dat *C. albicans* stikstofverbindings assimileer wat deur die afbraak van vleiland plantmatriaal vrygestel word. Hierdie afbraakproses kom deurlopend in vleiland en rivieroewer habitatte voor en verskaf potensieel energie en voedingstowwe aan *C. albicans*, spesifiek in die anaerobe sones waar konjugasie moontlik kan plaasvind, en 'n nis gevestig kan word, soos aangedui deur die Plankenburg- and Dieprivier.

Motivation

The ascomycetous anamorphic yeast, *C. albicans* is a commonly occurring commensal of vertebrates, and a renowned opportunistic pathogen (Cafarchia *et al.*, 2006; Girishkumar *et al.*, 1999; Kumamoto and Vinces, 2005). Nosocomial infections leading to candidaemia, which is systemic and often lethal, are the primary risk imposed by this fungus on man (Horn *et al*., 2009; Indhumati *et al*., 2009; Klevay *et al*., 2009; Moran *et al*., 2010; Pappas, 2006). However, superficial candidiasis, although not life-threatening, is also an expensive problem in nosocomial environments and immunocompromised patients (Kurtzman and Fell, 2000; Sangeorzan *et al.,* 1994; Stevens *et al*., 1991; Zaoutis *et al*., 2005). A major challenge is the increasing rate at which *C. albicans* develops resistance to antifungal treatment, due to its asexual adaptability (Navarathna *et al*., 2005; Sangeorzan *et al.,* 1994; Reboli *et al*., 2007; Redding *et al.,* 1994). Although parasexual conjugation has been demonstrated in *C. albicans* under anaerobic conditions, true meiosis has yet to be reported (Forsche *et al.*, 2008). Nevertheless, genetic diversity is maintained without meiosis, sufficient to allow the yeast to adapt its nutrient profiles by means of spontaneous mutation during lab maintenance (Ramirez and Lorenz, 2007; Rustchenko *et al*., 1994; Rustchenko *et al*., 1997). The parasexual cycle, non-disjunction and aneuploidy all contribute to the genetic diversity of *C. albicans* (Forsche *et al.*, 2008; Perepnikhatka *et al*., 1999; Rustchenko and Sherman, 2003).

One protective strategy of the human immune system is the diversity of microniches* within a single host. Pathogens and commensals are exposed to extreme environmental gradients within the human host, and adaptation to these numerous distinct microhabitats affords competitive strength. However, *C. albicans* has been demonstrated in almost every microhabitat in the human body; from commensal gastrointestinal tract residence to systemic bloodstream infections, and on surface habitats such as the mouth, vagina, skin and nose (Kumamoto and Vinces, 2005; Cafarchia *et al*., 2006; Kumamoto, 2008; Pappas *et al*., 2004). The adaptability of this yeast is demonstrated by its niche-specific gene expression

*Note: Defining and separating the terms ‘niche’, ‘microhabitat’ and ‘microniche’ are difficult tasks (Kearney, 2006; Kulesza, 1975; Miklos, 1959; Whittaker *et al.*, 1973). A niche is generally defined in terms of the function of an organism in relation to its neighbours within the ecosystem, whereas a microhabitat is rather limited to physical parameters. For the purpose of this study the words will be used interchangeably, describing an environment (both in terms of physical parameters and community interactions) in which the organism produces both its reproductive structures and assimilative thallus and generates its progeny (De Hoog *et al*., 2000) and simply where it can grow, rather than survive. This study focussed primarily on physical parameters, but using ‘niche’ and ‘microhabitat’ interchangeably emphasises the importance of the surrounding community, and encourages further consideration of this important pillar within the concept of an ecological niche.

within the host (Calderone and Fonzi, 2001; Kumamoto, 2008; Muschlegel and Fonzi, 1997). The yeast relies on the differential expression of multi-gene families and multi-transcriptional factor families to approach the various challenges presented by the broad range of host microhabitats (Barelle *et al*., 2006; Kumamoto, 2008; Muschlegel and Fonzi, 1997). In addition, *C. albicans* seems to be able to overcome different immune responses stimulated by the yeast in various infection sites, demonstrating again the adaptability of this yeast, particularly within the environment of the human host (Cassone, 1993; Fidel *et al*., 1999; Han *et al*., 1998).

The specific adaptation of *C. albicans* to the mammalian host has been repeatedly emphasised and environmental and clinical studies have generally assumed that its niche is limited to this physiological environment (De Hoog *et al.*, 2000; Fonzi *et al.*, 1993; Hube, 2004; Kerridge, 1993; Rooney and Klein, 2002). A number of authors have suggested that *C. albicans* may survive for long periods of time outside its vertebrate niche (Anderson, 1979; Crow *et al.,* 1977; Kacprzak and Stanczyk-Mazanek, 2003), however this has been overwhelmingly contended (Bernhardt *et al*., 1995; Hube, 2004; Marino *et al.*, 1995; Peter and Peter, 1988). The risk of infection from severely polluted environments has remained the predominant concern in terms of environmental studies (El-Taweel and Shaban, 2001; Philipp, 1991). The exposure of *C. albicans* to the external environment is largely due to sewage effluent, as its residence in the human gastrointestinal tract renders it a common inhabitant of wastewater (Arvanitidou *et al*., 2005; Kumamoto and Vinces, 2005; Vogel *et al.*, 2007). For instance, the *C. albicans* population size in sewage-contaminated rivers was shown to correlate with the coliform population size (Efstratiou *et al*., 1998), and it was suggested that the yeast could serve as a faecal indicator in contaminated waters (Elliot and Colwell, 1985). Wetland systems are regularly implemented in the removal of both pathogenic and organic loading in such contaminated waters and sewage effluents (Carleton *et al.,* 2001; Gerba *et al*., 1999). Interestingly, the oxygen-limited, reducing, plant debris-rich zones of wetlands and river banks are comparable to the anaerobic habitat of the human gastrointestinal tract (Hentges, 1993; Nelson *et al*., 2003; Seybold *et al*., 2002). In addition, oxygen-limitation is the only condition which was shown to stimulate the conjugation of *C. albicans* (Forsche *et al.*, 2008). This is of particular relevance, since De Hoog and colleagues (2000) suggested that the predominant conditions in which both the assimilative thallus and conjugation occur, is an important factor to consider in determining the possibility of an external niche for a human commensal or pathogen.

The risk of external *C. albicans* infection has been established in severely polluted regions, particularly in populations with a high incidence of HIV/AIDS (Philipp, 1991). The mortality rate of candidaemia in immunocompromised groups is approximately 40 % (Girishkumar *et al*., 1999; Horn *et al*., 2009; Indhumati *et al*., 2009; Klevay *et al*., 2009; Moran *et al*., 2010; Pappas *et al*., 2003; Pappas, 2006). In a South African context, populations with a high rate of HIV/AIDS infections frequent rivers for every-day water related tasks (Paulse *et al*., 2010). An external niche for *C. albicans*, harboured in wetland and river bank environments, would compound the risk of external infection by this opportunistic pathogen, particularly in these vulnerable population groups.

Based on the above observations, this study aimed to evaluate oxygen-limited zones in wetlands and river banks as a potential niche for *C. albicans*, since conditions in these zones are similar to the gastrointestinal niche of sewage-borne *C. albicans*. The first objective was to demonstrate the presence *C. albicans* in river bank and wetland environments, particularly comparing its abundance and survival in various zones. These zones were characterised and contrasted as (a) oxygen-limited, reducing and (b) aerobic, non-reducing zones. The second objective involved employing *in vitro* microcosm studies to determine the survival and growth of *C. albicans* in environments similar to those comprising the various zones of wetland and river bank habitats. Firstly, we aimed to determine the possibility of *C. albicans* survival in the rhizosphere, a capability that was previously demonstrated in yeasts (Botha *et al*., 2006; Cloete *et al.*, 2008). The survival within this habitat was compared to that of *C. albicans* in various soil types and on decaying plant matter. The final objective involved assessing the contribution of the components of decaying plant matter as a carbon source, within the context of established knowledge of the carbon utilisation profile of *C. albicans* (Kurtzman and Fell, 2000) and its confirmed adaptability to diverse nutrient sources (Rustchenko *et al.*, 1997). The particular carbon sources evaluated included cellulosic substrates and mono- and disaccharides released by the natural degradation of lignocellulosic wetland plant matter in anaerobic zones.

Acknowledgements

This work would not have been possible without the generosity of countless people. To those listed here, and those who helped in smaller ways, I am ever grateful.

Firstly, for the privilege of the ultimate adventure, guidance and grace:
'The heavens declare the glory of God;
The skies proclaim the work of His hands.
Day after day they pour forth speech,
Night after night they declare knowledge.' – Psalm 19:1.

My parents, sister, cousins and friends – the support and inspiration of loved ones are invaluable.

Prof A. Botha – for guidance, wisdom, creativity and, above all, for all of the opportunities and challenges. 'Ten minste is die lewe altyd interessant.'

The Botha Lab – for a lively atmosphere, and all their guidance; everything learnt and everything experienced.

The Medical Research Council – for the generous donation of all the clinical strains studied in this work.

Annatjie Hugo – for her assistance and guidance with the HPLC analyses.

Fletcher Hilten – for putting in more effort than necessary in assisting with the GC-MS analyses.

The National Research Foundation (NRF), the Water Research Commission (WRC) and the University of Stellenbosch – for the financial support that made this research attainable.

"Nature will bear the closest inspection.

She invites us to lay our eye level with the smallest leaf,

and take an insect view of the plain."

Henry David Thoreau

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Chapter One

Literature Review

1.1 A Basic Acquaintance with *Candida albicans*

1.1.1 The Yeast

The genus *Candida* is an artificial, polyphyletic taxon used to accommodate unrelated anamorphic ascomycetous yeasts lacking distinctive features needed for a more natural classification (Tsui *et al.*, 2008). The most well-studied representative of this genus is *Candida albicans* (Robin) Berkhout (1923) (Kurtzman and Fell, 2000). This yeast has no known sexual state, although mating was recently shown to occur under anaerobic conditions (Dimitru *et al.*, 2007) via a parasexual pathway (Forche *et al.*, 2008). *Candida albicans* is a common mammalian commensal that has generated interest due to its success in exploiting any opportunity to become the dominating species of its host (Girishkumar *et al.*, 1999). It resides as part of the normal flora in the gastrointestinal and genitourinary tracts of birds and mammals, as well as on the skin to a lesser extent, and is likely to occur wherever a mucous membrane occurs (Buck, 1990; Cafarchia *et al.*, 2006; Kumamoto and Vinces, 2005; Odds, 1988). It is also classified as an endosaprobe because of its ability to live and propagate in the digestive tract (De Hoog *et al.*, 2000; Kumamoto and Vinces, 2005). However, this dimorphic yeast is also the most frequent human pathogen of the genus *Candida*; narrowly followed by its close relative, *Candida dubliniensis* (Kurtzman and Fell, 2000). *Candida albicans* is viewed, along with the basidiomycetous yeast *Cryptococcus neoformans*, as one of the two principle yeast pathogens of mammals, leading to the substantial amount of literature on this organism. Although grouped with *C. neoformans* as a principle pathogenic yeast, *C. albicans* is distinct amongst yeast pathogens, as it has no known external environmental niche outside its human host (Hube, 2004; Kerridge, 1993).

1.1.2 Dimorphism

Although *C. albicans* is often referred to as a dimorphic yeast, its morphological variability is in reality vastly greater than that of other yeasts. It can grow vegetatively as a yeast but may also form pseudohyphae and true hyphae (reviewed by Berman and Sudberry, 2002; Sudberry *et al.*, 2004). Morphogenesis is complemented by phenotypic switching, a reversion of the yeast between spheroidal, white cells and ellipsoidal, opaque cells (Ramirez-Zavala *et al.*, 2008). This phenomenon, known as white-opaque switching, can be stimulated environmentally particularly under anaerobic conditions. The opaque morphology is significant, as it has been shown to facilitate the elusive conjugation of *C. albicans* under anaerobic conditions. In addition, *C. albicans* produces conidia, by budding from

single cells or from the mycelium (Berman and Sudberry, 2002); blastospores, notably the adherent basis of biofilm formation (Chandra *et al*., 2001a); and chlamydospores, also associated with the other mammalian-associated *Candida* species, *C. dubliniensis* (Staib and Morschhauser, 2006). *C. albicans* colonies can also show phenotypic variation (smooth or crenulated) on different media, a phenomenon probably related to the genetic instability of this yeast species (Berman and Sudberry, 2002), discussed later in this review.

Intuitively, such morphological variation should increase the survival of *C. albicans* in the diverse microniches of its host and is likely to facilitate the evolutionary success of the yeast as a commensal and opportunistic pathogen. This theory is supported by the diversity and complexity of the signal transduction pathways involved in phenotypic switching (Liu, 2001). The yeast-hyphal switch is regulated by a quorum-sensing mechanism and the signal involved has been identified as E,E-farnesol (Hornby *et al*., 2001; Nickerson *et al*., 2006). This extracellular quorum-sensing molecule prevents both yeast-to-hyphae conversion and aerobic mating. It achieves this using an inoculum size-effect, with mycelia evident after inoculating with $<10^6$ cells/mL and the yeast form evident after inoculation at $\geq 10^6$ cells/mL. Tyrosol, another quorum sensing molecule produced by *C. albicans*, stimulates hyphal growth and morphogenesis at low cell densities, but has not been explored as thoroughly as farnesol (Nickerson *et al*., 2006). In addition, farnesol prevents the formation of biofilms under certain conditions (reviewed by Nobile and Mitchell, 2006).

The ability of *C. albicans* to form biofilms is a serious nosocomial problem, particularly in terms of chronic indwelling medical devices (Chandra *et al.*, 2001a; Green *et al.*, 2004). These biofilms are heterogeneous; containing organised regions of blastospores, metabolically active cells and extracellular carbohydrate materials. They protect the cells from antifungal agents, as well as providing an ideal anaerobic niche for potential conjugation (Chandra *et al.*, 2001b; Dimitru *et al*., 2007; Nett *et al*., 2007).

It was speculated that, unlike biofilms, chlamydospore formation may be exclusively involved in *Candida* survival within the host, since the only two chlamydospore-producing *Candida* species, *C. albicans* and *C. dubliniensis*, are associated with warm-blooded animal hosts rather than the external environment (Staib and Morschauser, 2006). However, although these structures are universally associated with pathogenic *Candida* species, chlamydospores are rarely encountered in direct association with infection (Nobile *et al*., 2003), and may rather be involved in the survival of these two

host-adapted species when exposed to the harsh environment outside the host. Chlamydospores are three to four times the size of a yeast cell, and are spheroidal and thick-walled, often produced at the ends of filaments on nutrient-poor media. Although the significance of these spores is indicated by their universality among clinical isolates, their function remains a mystery.

Such morphological variation and the ability to form protective survival structures, the function of which remains undetermined, afford *C. albicans* adaptive advantages. This supports the contention that, if exposed to the appropriate environmental conditions, this yeast may be capable of surviving the external environment long enough to establish a niche away from its human host.

1.1.3 Mating

Until recently, it was thought that *C. albicans* was incapable of mating (Dimitru *et al*., 2007). However, it was demonstrated that the white-opaque switch facilitates mating of *C. albicans* under anaerobic conditions; whilst the quorum-sensing molecule, E,E-farnesol, represses mating under aerobic conditions. Thus, it was suggested that conjugation only occurs within the anaerobic regions of the gastrointestinal tract and within biofilms. Although this discovery was a breakthrough, it later arose that a parasexual cycle occurs during mating and true meiosis remains elusive (Forche *et al*., 2008). Conjugation between diploid a and α strains was shown to result in tetraploid strains which, instead normal meiotic separation, undergo efficient random chromosomal loss. This mechanism generally results in diploid strains, but imprecision frequently leads to aneuploidy. Random chromosomal loss in *C. albicans* is hypothesised to be involved in generating increased genetic diversity, since aneuploidy has been associated with phenotypic variation (Chen, 2007). In addition, ascospores which are traditionally associated with meiosis are highly antigenic. Therefore, the parasexual cycle affords *C. albicans* the benefits of genetic variation, whilst bypassing the formation of antigenic sexual spores. Although parasexual mating of *C. albicans* does produce significant genetic variation, this yeast is notorious for its ability to produce variation spontaneously, due to genetic instability.

1.1.4 The Genetic Landscape

The obscurity of the haploid phase of *C. albicans* hindered genetic studies and codon bias (a non-canonical genetic code in which CTG codes for serine instead of leucine) added a further obstacle to genetic manipulations (Cormack *et al*., 1997; Jones *et al*., 2004; Santos *et al*., 1993; White *et al*., 1995). Nevertheless, the genome of *C. albicans* was sequenced as a heterozygous diploid, facilitating the use of genetic manipulation for the study of this organism (Jones *et al.*, 2004). Whole genome comparisons between *C. albicans* and *Saccharomyces cerevisiae* showed some striking differences, particularly in the genetic basis of oxidative and sulphur metabolism. However, the two most relevant aspects of *C. albicans* revealed at a molecular level, in terms of our research, are its genetic instability and niche-specific gene expression.

Genetic Instability

Rustchenko and Sherman (2003) thoroughly reviewed the phenomenon of genetic instability in *C. albicans*. It seems that this yeast is able to maintain a high level of genetic variability without meiosis, or even conjugation. Therefore, natural diversity amongst the strains is conspicuous, even though mating is thought to be a relatively rare occurrence. Chromosomal rearrangements occur readily in *C. albicans* and are potential mechanisms for generating genetic diversity (Chen *et al*., 1998). For example, retrotransposons influence genetic diversity by gene inactivation, insertions, deletions and altered transcriptional control. A full-length retrotransposon, pCal1, has been identified in *C. albicans* along with a number of retrotransposon-like elements (Matthews *et al*., 1997), suggesting the ability to generate diversity in this manner. In addition, Perepnikhatka *et al*. (1999) showed the significance of asexual non-disjunction to adaptation. Negative and positive regulators control beneficial genes, and were shown to be controlled by changes in chromosome numbers via non-disjunction. A comparison of a large number of *C. albicans* strains showed notable variability in every chromosome (Rustchenko and Sherman, 2003). Even ordinary laboratory maintenance and sub-cloning causes significant chromosomal and ploidy alterations, particularly with regard to a small chromosome designated chromosome R. It was demonstrated that alterations in chromosome R alone can have a distinct influence on colony morphology. Environmental factors such as UV radiation, temperature, colony age, pH and immune serum were all shown to influence genetic and phenotypic instability.

Important physiological functions, including the utilisation of secondary nutrients, are associated with asexual chromosomal instability and chromosomal alterations (Rustchenko *et al*., 1994; Rustchenko *et al*., 1997). This supports the theory that genetic instability evolved as an alternative to meiosis in order to facilitate genetic variation. A study evaluated the utilisation of 21 carbon sources and three nitrogen sources at three different temperatures in more than 100 spontaneous mutants generated by laboratory maintenance of a single *C. albicans* strain (Rustchenko *et al*., 1997). It revealed that nutrient utilisation profiles of the mutants differed significantly to those of the parent strain. It seems that *C. albicans* may have the capability to produce enough genetic diversity via spontaneous mutation to cope with new environmental challenges. Ramirez and Lorenz substantiated this finding with similar results in 2007, suggesting that the regulation of alternative carbon metabolism in *C. albicans* is 'significantly different from that in other fungi'. Also, they demonstrated that a disruption of alternative carbon metabolic pathways attenuated virulence, highlighting the adaptive importance of this genetic variation.

In addition to such asexual chromosomal alterations, Forsche and colleagues (2008) showed that the parasexual cycle, characteristic of *C. albicans* during conjugation, is less stable than meiosis and also results in aneuploidy (1.1.3 Mating). Therefore, both asexual and parasexual aneuploidy and spontaneous chromosomal alterations increase the genetic variation and thereby the adaptability of this yeast within the human host. These mechanisms thus contribute to the capability of this organism to colonise a wide range of microhabitats.

Niche-Specific Gene Expression

The wide range of mammalian anatomical sites inhabited/infected by *C. albicans* is rare in a single species and can possibly only be rivalled by its oft-time neighbour, *Pseudomonas aeruginosa* (Calderone and Fonzi, 2001). *Candida albicans* has adapted to diverse site-specific pressures within its host and Kumamoto (2008) thoroughly reviewed this niche-specific gene expression. The review emphasises the diversity of environments that the yeast faces within a vertebrate, particularly the well-studied human host. A prime example of such adaptation was demonstrated by Muschlegel and Fonzi in 1997. They showed that *C. albicans* has two genes encoding two proteins involved in a single function in cell wall synthesis. The protein encoded by *PHR1* functions optimally at neutral pH. It is expressed in the neutral environment of the bloodstream and tissues, but is repressed under the acidic conditions of the vagina. In contrast, *PHR2*, encoding an analogous protein with an acidic optimum, has the inverse expression pattern contributing to the success of the yeast in both niches.

Another apt example of the niche-specific gene expression of *C. albicans* is the Sap family of isozymes (Kumamoto, 2008). Of the gene family encoding these enzymes, *SAP5* is expressed in the yeast in a range of sites within the host, *SAP2* is only expressed in *C. albicans* in the kidney, *SAP4* in the vagina and *SAP6* in yeast cells invading the reconstituted human epithelium (RHE) and human oral lesions. Similar differential expression of the *LIP* gene family (encoding extracellular lipase isozymes) has been demonstrated in both laboratory and clinical *C. albicans* strains. Also, Barelle *et al.* (2006) showed a notable heterogeneity of central carbon metabolic gene expression within a single focus of infection. Genes involved in glycolysis, gluconeogenesis and the glyoxylate cycle were found to be expressed simultaneously in a single infection site, demonstrating that a *C. albicans* population has the tools at hand to respond to a constantly altering environment (Barelle *et al*., 2006; Carman *et al*., 2008). This contributes to the biological fitness of the yeast within its host. It was also discovered that when nitrogen is limiting (when co-cultured with macrophages or neutrophils and whilst invading the RHE), genes involved in both amino acid biosynthesis and uptake are up-regulated (Kumamoto, 2008). In contrast, in the liver where nitrogen is in abundance, these genes are not up-regulated. In the micro-niches of the body where free radicals are used as defensive mechanisms, a host of genes are differentially up-regulated, encoding enzymes involved in detoxifying reactive oxygen (ROS) and reactive nitrogen (RNS) species. For instance, in neutrophils (where ROS are in abundance) genes such as *SOD* (encoding superoxide dismutases) and *CAT1* (encoding a catalase) are up-regulated and used to convert free radicals into harmless molecules. Flavohaemoglobins detoxify nitrogen free radicals, with the *YHB* gene family encoding these enzymes. These genes are up-regulated in the extremities where reactive nitrogen is used as a defence mechanism, but not in deep tissue infections where RNS are insignificant. Similarly, Calderone and Wadsworth (1993) described diverse, unique adhesins of *C. albicans* that each function in defined, separate regions within the host; another indication of niche-specific gene expression. Interestingly, it was found that *C. albicans* even shows plasticity in terms of respiration, with two alternative electron pathways providing another means of rapidly adjusting to altering environmental conditions (Helmerhorst *et al*., 2005).

In addition, another level of adaptability is afforded by multi-transcriptional factor families which differentially regulate the multi-gene families of *C. albicans* (Kumamoto, 2008). This ability to differentially control and optimise gene expression is what affords *C. albicans* its success as a host coloniser and pathogen. This could potentially also provide strong adaptive advantage in the environment external to the host.

The above-mentioned characteristics of this infamous ascomycete; including its morphological variation, reproductive trends, genetic instability and diversity of niches; describe an organism that is capable of responding to diverse environmental challenges with ease. Thus, exposure to an optimal environment external to its host may provide an opportunity for this yeast to adapt to a new environmental niche. This potentially poses a risk to human health, that of external infections by *C. albicans*, previously thought to be rare.

1.2. The Medical Approach

1.2.1 The Risk: Mortality, Epidemiology and Treatment

As repeatedly emphasised in this review, *C. albicans* inhabits virtually every microhabitat of the human body and therefore clinical manifestations involve virtually any organ (Pappas, 2006). It is a commensal inhabitant of the human host, found in more than 80 % of the faeces of healthy adult subjects (Kumamoto and Vinces, 2005). In addition, it colonises 7.1 % of newborn infants on the day of birth and 96 % of infants after one month, generally without any risk of infection. However, this commensal can become a clinical risk, particularly in response to immune deficiency. The major syndromes caused by *C. albicans* are summarised by Kurtzman and Fell (2000) as vulvovaginitis, dermatitis, cystitis, fever, myositis, hepatic dysfunction and mental confusion; all of which occur in varying combinations depending on the degree and site of infection. In any medical discussion of *C. albicans*, a distinction needs to be made between candidiasis; which includes superficial, largely mucocutaneous infections and candidaemia; an invasive, systemic infection associated with a high risk of fatality. Topical and systemic antifungal agents can be used to treat mucocutaneous candidiasis (Sangeorzan *et al.,* 1994; Stevens *et al*., 1991), but systemic bloodstream infections often cause death despite the use of antifungal agents and even treated candidiasis cases regularly recur (MacEntee, 1985; Redding *et al*., 1994; Pappas *et al*., 2009). The mortality rate of candidaemia has been reported between 20 and 70 %, with an average of approximately 40 % (Girishkumar *et al*., 1999; Horn *et al*., 2009; Indhumati *et al*., 2009; Klevay *et al*., 2009; Moran *et al*., 2010; Pappas *et al*., 2003; Pappas, 2006).

Candida infections are a prominent nosocomial concern, particularly in developed countries (Sternberg, 1994; Pappas *et al*., 2009). *Candida albicans* has become the fourth leading nosocomial

isolate in the United States and the most invasive mycosis in the developed world (Girishkumar *et al*., 1999). Ironically, medical progress nurtured the threat of invasive candidaemia. Fatal *C. albicans* infections are most often associated with life-prolonging developments; including catheters and other chronic invasive medical devices (Girishkumar *et al*., 1999; Nguyen *et al*., 1995), stem-cell transplants and cancer treatment (Bodey *et al*., 2002; Zollner-Schwetz *et al*., 2008), as well as antimicrobial treatments (Indhumati *et al*., 2009; Kurtzman and Fell, 2000; Navarathna *et al*., 2005). Fatality due to candidaemia is a constant threat in hospital Intensive Care Units (ICU; Nguyen *et al*., 1995; Ostrosky-Zeichner and Pappas, 2006). The financial burden of this disease is substantial, as it lengthens hospital stays and often demands repeated rounds of antifungal therapy. In 2005, Zaoutis and colleagues evaluated the cost per adult episode of candidaemia at $ 40 000 and similar financial and economic burdens are attributed to this nosocomial yeast by other authors (Miller *et al*., 2001; Moran *et al*., 2010; Vandijck *et al*., 2008).

Although attention is largely directed towards nosocomial candidaemia, superficial candidiasis is also regularly associated with malnutrition, pregnancy, HIV/AIDS infections, diabetes, malignant neoplasms and other diseases resulting in impaired immune systems (Calderone and Fonzi, 2001; Kurtzman and Fell, 2000; Sangeorzan *et al*., 1994). Interestingly, disseminated candidiasis, including vaginitis, has no definite association with HIV/AIDS alone and the immunological processes linking candidiasis and immune deficiency are not all well-established (Ampel, 1996; Imam *et al.*, 1990). However certain forms of candidiasis, particularly thrush, have often been linked to cellular immunodeficiency and thrush was even suggested as an indicator of immunodeficiency in AIDS patients (Calderone and Fonzi, 2001; Klein *et al.,* 1984; Sangeorzan *et al*., 1994).

In terms of epidemiology, candidiasis/candidaemia has few geographical limitations, since *C. albicans* is a mammalian commensal and humans have few geographical limitations (McCollough *et al*., 1999; Wilson and Plunkett, 1967). However, it is often associated with perspiration, and warmer climates are therefore conducive to the imbalance of this fungus in its host (Wilson and Plunkett, 1967). In regions of the world where the diets are high in carbohydrates, candidiasis cases are also more frequent. In addition, the occurrence of the fungus in the intestinal tract increases with age. Its incidence in hospitals is most renowned. Although literature reports an increasing trend towards other *Candida* species in nosocomial environments (Tortorano *et al*., 2004; Weinberger *et al*., 2005), *C. albicans* still dominates. *Candida albicans* represents approximately 40 to 60 % of nosocomial fungaemia cases and the fatalities linked to this species remain the highest (Girishkumar *et al.*, 1999; Horn *et al*., 2009;

Ostrosky-Zeichner and Pappas, 2006; Pappas *et al*., 2003; Reboli *et al*., 2007; Weinberger *et al*., 2005). Although the dominance of *C. albicans* in relation to other nosocomial *Candida* isolates is decreasing (reported at 70 % in a study spanning the 80's and 90's) it still remains the most prevalent nosocomial fungal threat. It also has an evolutionary advantage over its sibling species due to its long exposure to the human immune system, making it unlikely that the threat will diminish (Taylor *et al*., 1994; Tortorano *et al*., 2004).

The symptoms, cost and mortality associated with this opportunistic pathogen are dire, but the most threatening aspect is the rate at which *C. albicans* develops resistance to antifungal drugs (Sangeorzan *et al.,* 1994; Redding *et al.,* 1994). In addition, these drugs are not ideal as they are largely fungistatic rather than fungicidal, and may have deleterious side effects on the eukaryotic human host. Therapy for mucosal infections is dominated by the azole antifungal agents, predominantly fluconazole, which are considered safe and may be topically or systemically applied (Pappas *et al*., 2004). Invasive candidaemia calls for more vigorous and expensive treatment with rarer drugs, including amphotericin B-based preparations and echinocandin antifungal agents, in combination with the more common azoles. It has been unequivocally shown that exposure to sub-inhibitory levels of fluconazole increases yeast pathogenicity in *C. albicans* murine infections (Navarathna *et al*., 2005). This has been substantiated in humans by a report of a single patient needing increasing levels of fluconazole (ranging from 100 to 800 mg) to fight 14 infections by a single strain of *C. albicans* over a period of 2 years (Redding *et al*., 1994*).* Ultimately, fluconazole evoked progressively weaker responses and amphotericin B was employed to control the infection. *Candida albicans* strains resistant to such common treatments are increasingly reported (Melo *et al*., 2009; Perepnikhatka *et al*., 1999; Reboli *et al*., 2007). However, *C. albicans* attracts extensive research as a result of its mortality rate and financial burden, therefore progress is continually being made. New antifungal agents are regularly released, and research has shown that routine screening of hospitals and ICU wards lowers the fatality incidence of this highly adaptable yeast (Pappas *et al.*, 2009; Tortorano *et al.*, 2004). Understanding the threat of this organism and developing new antifungal agents involves a deeper understanding of the virulence factors that allow *C. albicans* to become aggressive toward its host.

1.2.2 Virulence Factors

Virulence factors are properties that enhance the penetration and survival of a pathogen in its host within the scope of an ecological strategy. These are in contrast to vitality factors like melanin or carotene, which are unspecific responses to harsh environments (Morschhauser *et al*., 1996). However, studying the virulence factors of a largely benign commensal like *C. albicans* is somewhat incomprehensive (Fidel *et al*., 2004; Kerridge, 1993). Since a compromise of host defences is the primary stimulus for *C. albicans* infection, changes in the host should be emphasised as much as fungal properties when studying this infectious agent. Even before the AIDS pandemic and the accompanied explosion of interest in compromised immunity, it was well-known that *C. albicans* is associated with other diseases, especially diabetes:

"There can be no suggestions for the reliable differentiation of disseminated candidiasis from other diseases, because it is always associated with one or more of the latter… It cannot be overemphasised that some other pathologic condition is always associated with moniliasis (*candidiasis*)… the presence of *Candida* should immediately inaugurate an intensive search for other such factors" (Wilson and Plunkett, 1967; *italics added*).

Nevertheless, treatment demands understanding the means of attack of the fungus, therefore the virulence factors of *C. albicans* have been explored and debated extensively. The pathogenic versatility of this yeast can be attributed directly to its virulence factors, even whilst infection generally cannot be separated from weakened immunity. Therefore, when exploring the infection mechanisms of *C. albicans*, it is vital to focus both on the human host and the yeast.

Virulence: The Human Immune System

As mentioned previously in the text, *C. albicans* is capable of inhabiting and therefore attacking a broad range of diverse niches within the host (Calderone and Fonzi, 2001). Thus, the study of the involvement of the immune system in candidiasis/candidaemia has been progressive, yet disparate. T-cell immune responses are often associated with oral pharyngeal, cutaneous and vaginal candidiasis; whereas neutrophils and mononuclear phagocytes (making up the functional phagocytic response) are mostly involved in systemic infections. The recurrence of certain *C. albicans* infections, specifically mucocutaneous infections and thrush, have been reported to increase proportionately with a CD4+

T-cell count decrease in AIDS patients (Imam *et al*., 1990; Ampel, 1996; Fidel, 2006). As mentioned earlier, under the heading '1.2.1 The Risk: Mortality, Epidemiology and Treatment', candidiasis has even been suggested as an indicator of AIDS infections, especially in certain population groups (Klein *et al.*, 1984). However, it seems other *C. albicans* infections, such as vaginitis, are not linked to AIDS, and therefore CD4+ T lymphocytes are not enlisted in the immune response against all *C. albicans* infections (Imam *et al.*, 1990). Humoral immunity is potentially also involved, since antibodies are effective in several animal models (Han *et al*., 1998). In addition to the complex combination of immune responses triggered against this well-adapted organism, it has yet to be determined which of the virulence factors are involved with the isotropic or apical stages of this polymorphic yeast. Cassone (1993) emphasised the dual nature of *Candida*-host interactions, and indicated that natural and acquired immunity need to interact to keep *C. albicans* infections at bay. Individual immunity mechanisms, often specifically combined, are needed to fight infection at each unique niche since *C. albicans* is uniquely adapted to each niche (Kumamoto, 2008). There is evidence that immune protection against the invasion of *C. albicans* is site-specific. Candidiasis models in animals showed that protection against systemic invasion does not protect against vaginitis (Fidel *et al*., 1999). Therefore, the defence mechanisms are as diverse as the niches, giving an indication of the difficulties involved in elucidating the mechanisms of defence against this versatile pathogen.

Virulence: The Yeast

The virulence factors of *C. albicans* are numerous, due to the wide spectrum of distinct anatomical invasion sites (Calderone and Fonzi, 2001). The virulence of *C. albicans* is largely due to its propensity to compete against mucosal microbiota and adapt to site-specific pressures within its host. Prime examples of this are the isozymes described previously in this review, under the heading '1.1.4 Niche-Specific Gene Expression' (Kumamoto, 2008; Muschlegel and Fonzi, 1997). They allow the yeast to adapt to the wide variety of distinct anatomical environments, which is a protective strategy of the host. Therefore, niche-specific gene expression is arguably the most effective virulence factor of *C. albicans*.

In addition, common virulence factors include farnesol production (Navarathna *et al*., 2005), adhesins (host recognition biomolecules), enzymes involved in morphogenesis, as well as the secretion of hydrolytic enzymes such as phospholipases and aspartyl proteases (Calderone and Fonzi, 2001). Phenotypic switching triggers changes in antigen expression and tissue affinities. Phospholipases increase the adherence ability of the yeast to animal cells, leading to host cell membrane damage and a

higher mortality rate in animal models (Da Costa *et al.,* 2009). Aspartyl proteases are involved in the degradation of both mucosal and immune components. In addition, *C. albicans* was found to increase virulence by producing prostaglandins that modulate the immune response (Noverr *et al*., 2001). Although morphogenesis is often considered a virulence factor, this remains contentious (Gow *et al*., 2002). According to clinical dogma, the dimorphic nature of *C. albicans* is thought to facilitate its opportunistic pathogenicity, allowing it to respond to an immune deficiency in its host by switching from its commensal yeast morphology to its pathogenic filamentous morphology (Fu *et al.*, 2002; Li *et al.*, 2002; Rocha *et al.*, 2001). However, as thoroughly argued by Romani *et al.* (2003) and Rooney and Klein (2002), this issue is debatable and morphogenesis has yet to be unequivocally established as a virulence factor of *C. albicans*. Although gene knockout studies and phase-locking of *C. albicans* in both the yeast and the hyphal form have repeatedly resulted in virulence repression, the mutations analysed could exhibit pleiotropic effects, influencing cell wall structure or the expression of other genes involved in virulence. It is therefore a strongly supported, but as yet unproven hypothesis that morphogenesis is a virulence factor. Even the concept that the hyphal form is the virulent form is debated. The hyphal form has often been found at infected sites, and mutants unable to form hyphae are less virulent and not often isolated from mammalian hosts (Rooney and Klein, 2002). However, mutants that are unable to take on the yeast morphology are also less virulent, and therefore the role of the yeast morphology in infection should not be underestimated. It is speculated that the yeast form is crucial for dissemination through the bloodstream (Rooney and Klein, 2002), whereas the mycelial form produces proteins (adhesins) crucial for yeast adhesion to endothelial cells (Hoyer, 2001), as well as compounds that protect the yeast against neutrophil killing (Smail *et al*., 1992) and macrophage-mediated phagocytosis (Borg-von Zeppelin *et al*., 1998). Finally, yeast-to-hyphal transition stimulates *C. albicans* phagocytosis by and the subsequent destruction of endothelial cells, providing an escape mechanism from the bloodstream (Phan *et al*., 2000). In essence, all morphologies and even transitions between morphologies, contribute to the effective invasion strategies of this organism.

Although the above-mentioned invasion capabilities of *C. albicans* were extensively studied, similar to the role of the human immune system during infection by this yeast, both are still classified as uncharted territories due to the ability of the organism to inhabit almost any niche in the host using diverse attack strategies. These diverse attack strategies emphasise the adaptability of *C. albicans*, and indicate again that the yeast should be able to respond and adapt to challenges faced in the environment external to the human host.

1.2.3 Evolutionary Champion

From the preceding information, it should now be evident that this literature review explores the possibility of a new or undiscovered niche in the environment for *C. albicans,* due to pollution. However, as also pointed out above, this yeast is primarily a human commensal, well-adapted to the specific niche of the mammalian host. It has rarely been found to be thriving in the external environment, and its survival may be negatively influenced by this specificity of adaptation to its host niche. To further evaluate the potential of *C. albicans* as a yeast adapted to both its host and the external environment, it is necessary to study literature on the evolution of pathogenic fungi, including this yeast.

In their discussion of the natural ecology of pathogenic fungi, De Hoog *et al*. (2000) describe a well-evolved pathogen as an organism that has adapted to coexist with its host without eradicating the host too quickly, which would abruptly end the chance of pathogen propagation. They suggest that more effective evolutionary adaptation involves reduced virulence, whereas lower degrees of adaptation are often evidenced by vigorous inflammation and disease. The highest degree of adaptation of an infectious agent is considered to be the ability to coexist with the host during the entire life cycle, as in the case of *C. albicans*. In the same discussion, they reiterate the fact that most fungi have an ecological niche external to the human host, implying that fungi are not generally well-evolved pathogens:

"Despite the fact that nearly all fungi do occur in the environment…"
"In medical mycology, many authors deny preferred human association in claiming that immunocompromised patients may be invaded by practically any fungus (Khardori, 1989)…"
"…some fungi have the ability and even preference to make use of the mammalian body during part of their life cycle, but nevertheless they probably all are able to grow and reproduce in the environment…"
"Fungi are therefore at most facultative pathogens…"

However, *C. albicans* seems to be an exception to this apparently common assumption, with numerous studies indicating that it is found exclusively in the human body and has a definite "preferred human association". As an example of such specific adaptation, Fonzi *et al.* (1993) describe a reduction of the transcription of Tca1 (a 5.5 kb retrotransposon-like element) in *C. albicans* 20- to 30-fold, when grown

at 37 ℃ relative to 25 ℃. This phenomenon was hypothesised to be related to the virulence of *C. albicans*, since cells grown at 37 ℃ were demonstrated to be less virulent than those grown at 25 ℃ (Antley and Hazen, 1988; Chen *et al.*, 1998). They suggested that the transposition of this retrotransposon-like Tca1 element is the reason for the variability of virulence between natural strains. In addition, and more relevant to our study, they hypothesised that, similar to the Ty elements of *S. cerevisiae*, the Tca1 elements may place adjacent genes under temperature-regulated control. This could lead to increased expression of survival genes at 25 ℃, allowing *C. albicans* to respond to the challenges external to its niche. Also, it could trigger the down-regulation of virulence genes at 37 ℃, facilitating a commensal rather than a parasitic relationship within its host. It may thus provide the yeast with the means to rapidly alter (decreasing, rather than increasing) its virulence within the host, thereby improving its evolutionary success (Chen and Fonzi, 1992).

There are various other indications that support the specific adaptation of *C. albicans* to coexist with the human/vertebrate host, as well as its uniqueness amongst fungal pathogens. For instance, according to Kerridge (1993), all systemic fungal pathogens except *C. albicans* infect via the lung. In contrast, *C. albicans* systemic infections occur when the yeast form moves from the gastrointestinal tract into the circulatory system. According to Wilson and Plunkett (1967), although obligate plant pathogens exist in the fungal kingdom, no known obligate human pathogens belong to this kingdom. In the same text, *C. albicans* is again listed as the exception. They mention that a large majority of pathogenic fungi are soil saprophytes and are unable to penetrate the skin, but must be inhaled or introduced via foreign particles (ie. thorns or splinters). The dermatophytes, which infect skin, hair and nails; and *C. albicans*, which can attack water-soaked skin and mucous membranes, are noted exceptions.

Another indication that *C. albicans* evolved within the human host is the response of this yeast to human hormones. A review by Rooney and Klein (2002) described the pronounced influence that human hormones have on morphogenesis. In addition, the exposure of *C. albicans* to 17-β-estradiol was shown to improve yeast growth and to enhance the expression of the multi-drug resistance marker CDR-1 (Zhang *et al.*, 2000). The complement receptors of this yeast are also strikingly similar to mammalian complement receptors (Hostetter *et al.*, 1993). These authors found that *C. albicans* contains surface proteins that bind C3D and iC3b, components of the immune complement system, an important part of innate immunity. Although other yeasts (including *S. cerevisiae*) have similar surface proteins that are able to bind complement, the surface proteins of *C. albicans* are much closer in structure to the mammalian complement receptors. This has significant implications for evolutionary

biology and demonstrates that *C. albicans* has evolved to specifically occupy the niches associated with its mammalian host. In addition, it suggests that *C. albicans* may need specifically human factors to influence its morphogenesis to more virulent, resistant morphologies; which could negatively influence its survival external to the human host.

Candida albicans has also evolved to influence and respond to another organism that is often resident in the human host, again emphasising the specific adaptation of the yeast to this habitat. Research by McAlester *et al*. (2008) determined the effects of the signalling molecules of two commonly co-infecting organisms of the lungs on each other. A signalling molecule of *Pseudomonas aeruginosa*, homoserine lactone (HSL), prevents the conversion of *C. albicans* from yeast to fungal morphology. In turn, farnesol, the quorum-sensing molecule of *C. albicans,* prevents swarming of *P. aeruginosa*. It is likely that these cross-effects are due to the common 12-carbon structure of both molecules, and indicates that both organisms are well-adapted to co-inhabiting this specific, mammalian environment.

From the above, it is obvious that *C. albicans* has the distinctive characteristic amongst fungi of being particularly well-adapted to survival in its host and rarely reported as an external risk. Knowledge of the vast and distinct host-fungal interactions abounds, and *C. albicans* seems to be inseparable from this niche. Therefore the risk of an environmental niche for *C. albicans* has not received attention comparable to that of its clinical niche. However, ontogeny and epidemiology are crucial in gaining knowledge about pathogens and in preventing infections (Cronin *et al*., 2010). The approach to human protection against most fungal pathogens, such as *C. neoformans*, involves an extensive knowledge of their external reservoirs (Botes *et al*., 2009; Tekaia and Latge, 2005; VandenBergh *et al*., 1999). Since fungi are overwhelmingly reported as saprophytic and externally abundant, the possibility of similar external reservoirs of *C. albicans* needs to be addressed.

1.3. The Ecological Approach

1.3.1 Host-Free Survival: Sewage Treatment and Wetlands

Candida albicans, being a resident of the mammalian gastrointestinal tract (Kumamoto and Vinces, 2005), naturally occurs as a prominent potential pathogen in sewage sludge. *Candida albicans* populations were found to correlate with coliform populations, which are renowned for their presence in wastewater (Efstratiou *et al*., 1998). The numbers of *Candida* species in general, as well as specific *C. albicans* counts, seem to reach an average of *ca.* 400 cells/100 mL in raw sewage (Cook and

Schlitzer, 1981; Hagler and Mendonca-Hagler, 1981), but have been known to reach as high as 10^5 cells/100 mL (El-Taweel and Shaban, 2001). Similar to *Escherichia coli*, the yeast can be used as an indicator of faecal contamination (Elliot and Colwell, 1985). However, no single organism encompasses all the crucial characteristics needed to define an indicator organism and there are numerous authors that caution against using any one indicator to determine pollution/pathogen levels (Buck and Bubucis, 1978; Dionisio *et al.*, 2000; Elliot and Colwell, 1985). These authors all suggest *Candida* species as appropriate indicators to be used as a substantiation of other indicator organisms. Buck and Bubucis (1978) describe a feasible identification method for use in counting culturable *C. albicans* in natural waters. Also, quantitative Real-Time PCR (qRT-PCR) has been optimised in order to enumerate *Candida* species in water samples with ease (Brinkman *et al.*, 2003). These enumeration techniques have allowed investigations into the survival of *C. albicans* in environments external to the human host, and provided some clues as to the possibility of an external niche.

Despite the above-mentioned presence of *C. albicans* in wastewater, the die-off phenomenon (due to several abiotic factors) of telluric, allochthonous organisms casts particular doubt on most pathogens, including *C. albicans*, as indicator organisms (Borrego *et al.*, 1983; Rheinheimer, 1992). Borrego and his colleagues (1983) describe the quick dilution of pathogens once exposed to the ocean; as well as other lethal factors such as nutrient limitations, solar radiation, temperature variation, phages, predation, and high concentrations of inorganic salts and heavy metals. All of these could negatively affect the survival of *C. albicans* once exposed to the environment outside of its human host. They suggest the use of phages rather than bacteria or fungi as sewage indicators, since the survival of phages is generally longer than the survival of bacteria and eukaryotes. Confirming this die-off phenomenon, *C. albicans* has been reported as a low-incidence yeast in polluted natural waters (Marino *et al.*, 1995). Nevertheless, *C. albicans* is regularly isolated during studies monitoring faecal contamination of drinking water, recreational beaches (sand and water) and rivers across the globe; and is generally the most prevalent yeast species in these contaminated waters (Arvanitidou *et al.*, 2005; Buck and Bubucis, 1978; El-Taweel and Shaban, 2001; Vogel *et al.*, 2007). In addition, *C. albicans* has been associated with skin infections due to recreational activity in sewage-contaminated waters, emphasising the risk of human exposure to *C. albicans* in the external environment (Philipp, 1991).

A notable finding regarding the fate of pathogens in polluted waters was obtained by Matson *et al.* (1978). They demonstrated a significant increase in pathogen concentrations in sedimentary samples of contaminated waters, compared to subsurface water columns. These authors cautioned that due to

settling, the increase in concentration may not be attributed to a higher survival rate. However, they did show that many factors that contribute to the die-off phenomenon, such as dilution and exposure to UV radiation, have a decreased impact at sedimentary levels. This is significant in terms of yeasts as their physical size causes them to sink to the bottom of watery environments. The tendency of *C. albicans* to form biofilms is also noteworthy with regard to protection from environmental stress (Nobile *et al*., 2003). These studies mostly indicate that in faecally contaminated waters *C. albicans* is a potential risk to both healthy and immunocompromised individuals. However, although simple enumeration and water quality testing in polluted regions is common, true survival studies on *C. albicans* are sparse and ambiguous.

In contrast to reports of its presence in contaminated sites, reports of the true survival of *C. albicans* in soil, water and the rhizosphere remain contentious. Hube (2004) stated that *C. albicans* is rarely found in environmental habitats such as soil, unlike most other human pathogenic fungi, including *C. neoformans* (Casadevall *et al*., 2003), *Aspergillus fumigatus* (Latge, 2001) and *Histoplasma capsulatum* (Woods, 2003). In a review of the link between yeast morphogenesis and pathogenicity, Rooney and Klein (2002) emphasised the uniqueness of *C. albicans*; concluding that most fungi that fall into the pathogenic, dimorphic subgroup are found in the external environment as saprophytic moulds. In contrast, *C. albicans* has no established external niche and is therefore not considered an exogenous risk. Our discussion of its evolutionary success within the host (1.2.3 Evolutionary Champion) substantiates this. It is classified as an endosaprobe (De Hoog *et al*., 2000) because its ecological niche - the habitat from which the species is most often isolated - is limited to the human host. Even its survival in faeces outside the host is short-lived, shown to be due to its low competitive ability (Bernhardt *et al*., 1995). These authors revealed the major impact of residential intestinal flora on preventing the growth of *C. albicans* to infective proportions, both inside and outside the host. Therefore, although growth conditions within the intestine are ideal for *C. albicans*, its numbers are kept in check as a result of competition with other microbes (Kennedy *et al*., 1987). As mentioned by De Hoog *et al*. (2000), an evolutionarily successful host-associated organism is actually less pathogenic, in the case of *C. albicans* probably partly due to the competition described by Bernhardt *et al*. (1995) and Kennedy *et al*. (1987). This phenomenon may also reduce the chances of *C. albicans* survival in the natural environment away from its host.

Although the yeast seems inseparable from its vertebrate niche, according to Hube (2004) *C. albicans* has no nutrient requirements that should prevent it from surviving in the external environment, and it is

therefore surprising that this yeast has not developed an external niche as effectively as it has adapted to living in its host. From an evolutionary perspective, Bastidas and Heitman (2009) claim in their review that the formation of hyphae afforded *C. albicans* the ability to evolve into a successful commensal of the gastrointestinal tract. They suggest that this development allowed the yeast to compete with bacteria and survive when forming a cooperative multi-species biofilm and it should be capable of performing similarly externally. Nevertheless, Kurtzman and Fell (2000) classified it as an obligate commensal because of the rarity of its host-free occurrence, and it is generally accepted as such. However, *C. albicans* has on occasion been isolated from natural environments. For instance, an atypical *C. albicans* strain was isolated from the North Sea in 1977, with no evidence of pollution contamination (Crow *et al.,* 1977). The strain was thought to have originated from sea birds and became 'atypical' during adaptation to a novel environmental niche. *Candida albicans* was also isolated from apparently pristine river sites in 1987 by Valdes-Collazo and colleagues. They showed that *C. albicans* survives well in both fresh and marine pristine tropical waters, although much better in waters continually fed with sewage effluent. However, the experimental sites deemed pristine by these authors were in the vicinity of sewage effluents and this is likely to have influenced yeast survival. In 1988, the persistence of several pathogenic fungi in seawater was investigated under laboratory conditions and *C. albicans* demonstrated the shortest survival time of those species considered (Peter and Peter, 1988). It was also noted that the survival of *C. albicans* is dependent upon various external factors, particularly the associated flora. In contrast, Anderson (1979) found that *C. albicans* and a number of other pathogenic yeast can survive up to 52 weeks in salinities and temperatures exceeding those of the natural oceanic environment, concluding that seawater is a potential niche for pathogenic fungi such as *C. albicans.* Similarly, Kacprzak and Stanczyk-Mazanek (2003) reported the survival of *C. albicans* in soil, contaminated once-off with sewage, for over one year. It seems that although *C. albicans* may be capable of survival in some natural environments under specific conditions, the establishment of an ecological niche outside the mammal has yet to be conclusively demonstrated. The major concerns regarding infection from the natural environment have thus far been limited to severely polluted natural reservoirs. However, potential niche development in wetlands and rivers by *C. albicans* has yet to be evaluated as a concern.

Wetlands were found to be at least as successful as sewage treatment plants in the effective removal of pathogens, phosphate, ammonium and nitrate from wastewater (Gerba *et al*., 1999; Gersberg *et al.*, 1989). Storm-water runoffs can be treated with constructed wetlands using the same mass balance calculations as conventional wetlands (Carleton *et al.,* 2001). The major problem associated with the

use of wetlands to treat wastewater is the need for extensive areas of land with ideal vegetation. However, constructed/engineered wetlands, such as gravel-based wetlands, can assist in overcoming this problem (Werker *et al*., 2002; Yang *et al*., 2001). The benefit of natural wetlands is that sewage treatment and maintenance of natural vegetation can occur hand-in-hand (as evidenced in Zeekoeivlei, on the Cape Flats in the Western Cape of South Africa; where bird watching and sewage treatment occur within visible distance of each other). However, it is argued that wastewater significantly impacts the natural vegetation of wetlands, raw sewage having a much greater impact than secondary, preliminarily-treated sewage (Junk *et al*., 2006). In addition, wetland biodiversity is negatively impacted by the increasing demands of human wastewater overloads. Storm-water runoffs often result in raw sewage reaching inhabited, urban areas (Green and Martin, 1996). Wetlands can be used to treat such storm-water runoffs, but the pathogen and organic loads are much higher than in secondary wastewater. Bird migration has also been demonstrated as a significant problem, transferring pathogens from one wetland to another during wintering and stopover periods in different wetland regions (Jourdain *et al*., 2007). The Avian influenza and the Nile virus are examples of the increased transmission risk of bird-borne diseases introduced by concentrating vertebrate pathogens in wetland areas. Birds are established as a common reservoir of *C. albicans* (Buck, 1990; Cafarchia *et al.*, 2006) and this pathogen is also likely to experience increased transmission due to bird migration, especially if it becomes established in wetlands. This establishment is a feasible possibility, as wetlands and river banks provide the ideal plant debris-rich, anaerobic environment for saprophytic activity to occur (Seybold *et al*., 2002).

1.3.2 Host-Free Survival: An Oxygen-Limited, Reducing Environment

In considering the ability of a pathogen to establish itself in an exogenous habitat, a crucial factor to investigate is the specific conditions to which the pathogen has adapted in the well-studied environment of the host. As discussed in this review under the heading ‘1.2 The Medical Approach’, the niche of *C. albicans* is not defined but rather as broad and diverse as the conditions that make up the various microniches of the human body, both external and internal (Kumamoto, 2008). However, the fact that *C. albicans* was found to conjugate in an anaerobic environment, similar to that of the gastrointestinal tract, leads us to focus on those conditions as its optimal niche. In addition, the yeast is expelled into sewage streams directly from the gastrointestinal tract (Kumamoto and Vinces, 2005). Within sewage streams entering the anaerobic zones of rivers and wetlands, the yeast encounters oxygen limitation, a reducing environment and potential carbon sources released by plant matter (Seybold *et al.*, 2002). This

environment to which *C. albicans* is exposed along river banks and during wetland filtering of sewage, bears a striking resemblance to the gastrointestinal tract (Hentges, 1993; Nelson *et al*., 2003), and could harbour a niche for the external survival of this yeast.

In a fascinating discussion of the natural ecology of pathogenic fungi, De Hoog *et al*. (2000) define an ecological niche as the environment where the fungus in question produces both its assimilative thallus and sexual structures, generating its progeny. This is particularly relevant to *C. albicans*, whose parasexual cycle has only been described under anaerobic conditions (Dimitru *et al.*, 2007). This should have a significant influence on the discovery a new, or as yet undetected, environmental niche. De Hoog and colleagues also describe *Horteae werneckii*, an allochthonous human commensal that colonises the feet and hands. It was eventually shown that its natural habitat is particularly extreme: salt-saturated pools and tidal waters (Gunde-Cimerman *et al.*, 2000). Such resistance to osmotic stress allows the survival of these organisms on the vertebrate skin. They argue that this gives a strong indication that both the commensal and external niche could be determined by a single, or very few, pertinent characteristics. This relates intimately to our research on *C. albicans*, since the benefit of an anaerobic, reducing environment reasserts itself repeatedly in literature on the biology of this yeast.

1.3.3 Host-Free Survival: The Carbon Source

As it has been established in this review that a reducing, anaerobic environment may support the survival of *C. albicans* within wetland sewage treatment systems and river banks, an exploration of the major limiting factor - an energy or carbon source - should follow. Within wetlands and river banks, *C. albicans* is exposed to both live plant roots and dead plant matter as possible carbon sources. Yeasts have been shown to occur in mycorrhizal-like relationships with plants (Botha, 2006; Cloete *et al*., 2008), and the utilisation of sugars in the rhizosphere could stimulate the survival of *C. albicans* in anaerobic wetland zones (Farrar *et al*., 2003). However, since fungi are overwhelmingly saprophytic (Kerridge, 1993) and *C. albicans* is exposed to decomposing plant matter in the gastrointestinal tract (Hentges, 1993; Nelson *et al*., 2003), it is most likely that the yeasts will instead utilise carbon sources provided by decomposing plant matter. This anaerobic, reducing environment, rich in decomposing plant matter, is both a considerable component of the gastrointestinal tract and of the anaerobic zones of wetlands (Hentges, 1993; Nelson *et al*., 2003; Seybold *et al*., 2002). The theory that plant matter may play a role in the survival of *C. albicans* in rivers and wetlands is supported by Elliot and Colwell (1985), who showed that yeasts are primarily isolated from polluted oceans in association with

seaweed. The simple degradation products of lignocellulose are a likely carbon source for *C. albicans* (Saha, 2003; Van Uden, 1960), although the ability to utilise cellulose would provide the yeast with a competitive advantage in this environment. Evaluating the carbon utilisation capabilities of *C. albicans* involves studying the potential of this yeast to degrade cellulose (by both enzymatic and alternative means) as well as investigating its ability to grow on the natural degradation products of lignocellulose.

Investigating the possibility of lignocellulose utilisation, or the degradation products thereof, by *C. albicans* involves understanding the structure of plant matter. Cellulose is a linear polymer consisting of long chains of the repeating cellobiose unit, comprising two glucose residues rotated 180° relative to each other (Beguin and Aubert, 1994). The parallel chains, bound with hydrogen bonds, form microfibrils made up of ordered crystalline domains and less-ordered amorphous regions. The degree of crystallinity can vary, reaching up to 70 % in cotton. The role of cellulose is structural, giving plants mechanical strength to withstand osmotic stress. Cellulose carries out this structural function along with hemicellulose and lignin, combined to form a lignocellulosic matrix. A complex battery of enzymes is needed to degrade the hemicellulose in plants, as it is a heterogeneous polymer of pentoses, hexoses and sugar acids (Saha, 2003). However, natural degradation results in the release of simple mono- and disaccharides, and the specific pentoses and hexoses released depend on the composition of the hemicellulosic plant matter in question. In order for *C. albicans* to survive in the wetland environment, it must be able to utilise this lignocellulosic energy source or its natural degradation products.

There is little or no literature describing the survival of *C. albicans* in the rhizosphere or on substrates such as decomposing plant matter. Hunter and Fraser (1989), Rustchenko *et al*. (1997), Kurtzman and Fell (2000) and Chandra *et al*. (2001a) all independently recorded *C. albicans* as unable to utilise or assimilate cellobiose, which suggests that this yeast will not enzymatically utilise cellulose in any form in its metabolism. The degradation of cellulose and pectin has been demonstrated in yeasts (Dennis, 1972; Spindler *et al*., 1988) and this metabolic capacity would afford *C. albicans* an advantage in obtaining energy from wetland habitats. Although the enzymatic cellulose utilisation capacity of *C. albicans* has yet to be demonstrated, alternative methods could also be explored. For instance, *C. albicans* is known to lower the pH of its environment (Nikawa *et al*., 1994; Samaranayake *et al*., 2006) and may use acid hydrolysis to degrade lignocellulose to its glucose monomers. However, the most likely carbon source for *C. albicans* in the wetland habitat is mono- and disaccharides released by natural degradation of lignocellulose.

Literature has shown that the only enzyme isolated from *C. albicans* potentially involved in plant debris degradation is a β-1,3-exoglucanase (Chambers *et al*., 1993). This enzyme hydrolyses the β-1,3 bonds of β-glucans, a major constituent in yeast cell walls, as well as barley and oat grains. The β-glucans of most other plants contain predominantly β-1,6 bonds. As the β-1,3-exoglucanase is thus far the only enzyme isolated with a possible involvement in plant matter degradation, the establishment of the natural function of this enzyme is necessary. However, this enzyme is thought mainly to be involved in fungal morphogenesis and the degradation and synthesis of the fungal cell wall. As discussed earlier, *C. albicans* is a constantly morphing fungus, and such morphogenesis is dependent on lytic enzymes (Berman and Sudberry, 2002). Stubbs and colleagues (1999) showed that, in the presence of excess lamarin-containing substrates, the transglucosylation ability of the enzyme can be up to ten times faster than the hydrolytic capability of the enzyme, indicating that the primary enzyme function might lie in cell wall synthesis rather than degradation. Recently, other functions have been assigned to this enzyme, including cell separation (Esteban *et al*., 2005). It is unlikely to be involved in plant matter degradation due to the specificity of the enzyme for its substrate. In addition, more enzymes would be needed to contribute to the effective utilisation of lignocellulose by *C. albicans*.

Alternatively, *C. albicans* may release the degradation products of cellulose via acid hydrolysis, similar to a pretreatment process used in bioethanol production that involves dilute acid hydrolysis of lignocellulosic substrates (Lee *et al*., 1999; Torget *et al*., 2000; Saha *et al*., 2005). *Candida albicans* is renowned for acid production via glucose fermentation, particularly within biofilms in the oral environment (Nikawa *et al*., 1994; Samaranayake *et al*., 2006). The acidic metabolites produced include pyruvates and acetates, which lower the surrounding pH to as low as 3. This proton-rich environment has the potential to hydrolyse lignocellulose, releasing simpler degradation products and providing an ideal carbon source in the wetland environment. However, Samaranayake (1983) showed that this acid production does not occur under anaerobic conditions, and several studies indicated that optimum biofilm formation takes place under aerobic rather than anaerobic conditions (Biswas and Chaffin, 2005; Samaranayake *et al*., 1983; Thein *et al*., 2007). Therefore, this potential method of lignocellulose utilisation is more likely to occur under aerobic conditions, whilst our study is concerned with anaerobic environments.

Simple carbohydrates released from decaying plant matter are the most likely source of carbon and energy for *C. albicans* within the wetland habitat (Van Uden, 1960). Kurtzman and Fell (2000) classified *C. albicans* as a yeast able to grow aerobically on arabinose, glucose, galactose, maltose,

sucrose and xylose. They also recorded the ability of this yeast to anaerobically ferment glucose, as well as galactose and sucrose. However, this nutrient profile is not rigid, as found by Rustchenko *et al.* (1994) and Rustchenko and Sherman (2003). Natural plant degradation, which occurs within wetland and river bank habitats, releases a broad spectrum of such mono- and disaccharides (Saha, 2003). This degradation may thus provide the ideal carbon sources for *C. albicans* within the anaerobic, reducing wetland habitat.

From the above, it is obvious that decomposing plant matter in wetlands may provide carbon sources to *C. albicans*, analogous to the environment within the human gastrointestinal tract (Hentges, 1993; Nelson *et al*., 2003; Saha, 2003; Seybold *et al*., 2002). *Candida albicans* is thus potentially capable of utilising the carbon sources within the environment external to its host. The ability to degrade cellulose would provide the yeast with a competitive advantage in this situation, but would involve enzymes or acid hydrolysis of cellulose, both of which are unlikely. However, in this anaerobic environment, mono- and disaccharides are released during plant decay, which are a known nutrient source for *C. albicans* (Saha, 2003). In addition, *C. albicans* has the capacity to adapt its nutrient profile (Rustchenko *et al*., 1997) and sewage-borne *C. albicans* should be capable of using mono- and disaccharides to establish a microhabitat once exposed to the anaerobic wetland environment.

1.4. Conclusions: An External Ecological Niche for *C. albicans*

Candida albicans is a common human commensal that has been extensively studied from the medical perspective (1.2. The Medical Approach), but has received comparatively little attention regarding its ability to establish itself in a niche in the external environment away from its host.

It demonstrates morphological variation to an extent that is rare in yeasts (1.1.2 Dimorphism), and contains a substantial number of mechanisms for generating genetic diversity without meiosis (1.1.4 The Genetic Landscape: Genetic Instability). In addition, it inhabits and infects a broad range of host microhabitats and has distinct genetic responses in each unique microhabitat (1.1.4 The Genetic Landscape: Niche-Specific Gene Expression). This genetic plasticity allows for constantly altering nutrient profiles (1.1.4 The Genetic Landscape: Genetic Instability and Niche-Specific Gene Expression). Such characteristics describe a highly adaptable organism.

Candida albicans has been shown to conjugate exclusively in anaerobic environments (1.1.3 Mating), possibly in niches such as the gastrointestinal tract and within biofilms. When exposed to such anaerobic conditions outside of the human host, its above-mentioned adaptability may afford it the opportunity to establish a niche in the external environment. Competition (1.3.1 Host-free Survival: Sewage Treatment and Wetlands) and host-specific adaptation (1.2.3 Evolutionary Champion) are the predominant potential limitations to the external survival of this commensal yeast when exposed to optimal conditions. Wetland and river bank habitats are often employed in sewage treatment and receive a constant inflow of human commensals and pathogens such as *C. albicans* (1.3.1 Host-free Survival: Sewage Treatment and Wetlands). Conditions within these wetland and riverbank environments are comparable to those in the gastrointestinal tract (1.3.2 Host-free Survival: An Anaerobic, Reducing Environment), particularly in terms of oxygen-limitation, redox potential and decaying plant matter (1.3.3 Host-free Survival: The Carbon Source). These external habitats may therefore harbour a potential niche for this opportunistic pathogen.

In South Africa, a large immunocompromised population due to HIV/AIDS are often settled along river banks and in wetland areas (Paulse *et al*., 2009). The mortality rate of candideamia is 40 % (1.2.1 The Risk: Epidemiology, Mortality and Treatment) in such a population, which habitually uses this water for bathing, washing and drinking. This motivates the need to determine the possibility of a niche for *C. albicans* in anaerobic, reducing, plant debris-rich wetland and river bank zones.

1.5. Research Aims

Thus, with the above as background, the aim of this project was to establish the risk of *C. albicans* occurring in a niche, external to its human host within a wetland habitat. We planned to evaluate microhabitat preferences of *C. albicans* in natural river bank and wetland environments; particularly comparing anaerobic, reducing zones to aerobic, low-nutrient zones. In addition, growth and survival of *C. albicans* were studied in an *in vitro* laboratory environment simulating that of wetland habitats. The latter included the rhizosphere, mud, decaying plants and soil. The potential carbon sources in these habitats that we aimed to evaluate include those associated with decaying plant matter, such as cellulosic substrates and mono- and disaccharides released by the natural degradation of lignocellulose.

Chapter Two

Experimental Work

2.1 Materials and Methods

2.1.1 *Candida albicans* in the External Environment: Rivers and Wetlands

The Plankenburg River, flowing through Stellenbosch, South Africa, is approximately 10 km long and is characterised by an industrial area and an informal settlement along its banks, with an estimated population of 22 000 people and inadequate sewage facilities (Paulse *et al*., 2009). The sampling site (33°55'50"S, 18°51'10"E) was 1 km downstream of the informal settlement, but precedes the majority of the industrial effluent.

The Diep River, flowing through the outskirts of Cape Town, South Africa, is approximately 65 km in length surrounded by a 900 ha wetland conservation region, in turn surrounded by residential and industrial areas. The river is fed by the Potsdam Wastewater Treatment Works, and the sampling site (33°50'15"S, 18°29'15"E) was approximately 5 km downstream from this plant.

The Plankenburg and Diep Rivers were evaluated in this study; particularly comparing oxygen-limited zones rich in plant debris, as well as aerobic zones clear of plant debris in each river. The redox potential of the respective zones sampled was evaluated using a hand-held Waterproof pH/ORP Meter H1905 Microcomputer (Hanna Instruments). Correlations were drawn between the total yeast counts and the total coliform counts in each zone, to evaluate the levels of pollution in the river during the sampling period. Finally, both semi-quantitative plate count and quantitative RT-PCR methods were employed to compare the concentrations of *C. albicans* in the various zones of the rivers during the sampling period.

2.1.1.1 Sample collection

Samples were collected every monthly using a specialised stainless steel sampling device with a 1.5 m long handle, thoroughly sterilised before and after each sampling event. Samples of one litre per zone were taken from between 50 cm and 100 cm below the river surface level, stored in sterile 1 L jars and transported immediately on ice to the laboratory. The zones included: Clear, Flowing Water (W), Rock-Filtered Water (R) and Plant Debris-Filtered Water (P). Care was taken to sample from water that was not stagnant, particularly in the R and P zones. The rock-filtered zones were selected in order to

compare water that was also filtered (as with plants) but in the absence of oxygen-limitation and high levels of plant debris.

2.1.1.2 Correlation of coliform and total yeast counts

Coliform concentrations were determined by plating out 100 µL of each sample (W, R and P) in triplicate on McConkey agar (pH 7.1) and subsequently incubating the plates at 44 ℃ for 48 h (Atlas, 1993). After incubation, coliform colonies were identified by a metallic red hue and counted. Total yeast concentrations were determined by plating out 100 µL of each sample in triplicate on Sabouraud glucose agar (SGA, pH 5.6) supplemented with 600 mg/L chloramphenicol (Sigma, St. Louis, USA) and subsequently incubating the plates at 30 ℃ for 48 h (Atlas, 1993). After incubation, the total yeast colonies were counted. Correlations between the coliform and total yeast concentrations in each zone (W, R and P) were evaluated using Statistica Version 9.0 (Statsoft).

2.1.1.3 Semi-quantitative culture identification of *C. albicans* in environmental samples

One hundred millilitre aliquots of each sample (W, R and P) were filtered (0.45 µm pore size cellulose nitrate filter disks; Sartorius Sedim Biolab Products, Aubagne, France) in separate sterile polycarbonate filter systems (Sartorius Stedim Biolab Products, Aubagne, France) and the filter disks were transferred to SGA and incubated at 37 ℃ until yeast colonies had developed to a stage where they were clear, but not over-grown with filamentous fungi (approximately 24-48 h). Colonies were randomly selected and purified on yeast malt (YM, pH 5.0) agar supplemented with 600 mg/L chloramphenicol (approximately 60 colonies per sample). They were subsequently transferred to a commercial medium for *Candida* species differentiation, Candiselect4 (Bio-Rad, Marnes-la-Coquette, France), and were incubated at 30 ℃ for 24 h. Purple colonies were tentatively identified as *C. albicans*, whereas blue colonies represented *Candida tropicalis* and other yeasts were unpigmented. The identities of putative *C. albicans* strains were periodically confirmed by analysis of the D1/D2 and ITS gene regions as described in 2.1.2.2. The approximate percentage of *C. albicans* numbers relative to the total yeasts transferred from the filter in each zone was recorded after every sampling event.

2.1.1.4 Molecular quantification of *C. albicans* in environmental samples

Quantitative RT-PCR was employed to compare the concentrations of *C. albicans* in the respective river zones. Fluorigenic 5' nuclease chemistry, more commonly referred to as Taqman chemistry, was used; probes, primers, reference cells, standards and qRT-PCR parameters were according to Brinkman *et al*. (2003).

Sample DNA preparation

One hundred millilitre aliquots of each sample (W, R and P) were separately filtered, concentrating the yeasts on the filter disk. An adjustment to the protocol described by Brinkman *et al*. (2003) involved separate step-wise filtering of all samples, since plant debris remaining on the filter disks was inhibitory to RT-PCR reactions. Consequently, 100 mL aliquots of each sample were first alternately vortexed (maximum speed; Vortex Genie 2, Scientific Industries, NY, USA) and shaken for ten minutes prior to filtering in order to separate yeasts from particulate matter. The particulate matter was removed by passing the samples through a sterile sieve (0.1 mm pore-size), subsequently filtering through Whatman 1 filter paper (Schleicher and Schuell, New Hampshire, US) and lastly filtering (0.45 µm pore size cellulose nitrate filter disks) in separate sterile polycarbonate filter systems. Each filter was vortexed (maximum speed, 5 min) with sterile, acid-washed glass beads (Sigma, St. Louis, USA) in 1 mL physiological saline solution [PSS, 8.9 g/L sodium chloride (Kimix, Cape Town, RSA)]. The filters were removed and the Eppendorf tubes centrifuged in a bench-top centrifuge (Biofuge fresco, Heraeus Instruments, Hanau, Germany) at 5000 x g for 10 min. The supernatant was removed and the pellet resuspended in 100 µL PSS. The resulting suspension was submitted to a DNA extraction procedure using the Zymo Fungal/Bacterial DNA kit (Zymo Research, California, USA) according to manufacturer instructions.

Standard DNA preparation

Yeast inocula were prepared by culturing *C. albicans* CAB 628-1 at 30 ℃ in 250 mL conical flasks containing 30 mL YM broth on a rotary shaker (150 rpm, 25 mm throw) in the dark. The yeast cells, allowed to grow to log phase, were subsequently harvested via centrifugation at room temperature in 2 mL Eppendorf tubes (5000 x g, 10 min). The resulting pellet was resuspended in PSS. Yeast concentrations were determined microscopically using direct cell counts on a haemocytometer (Superior, Marienfeld, Germany), and diluted to the correct concentration in PSS. A concentration range of 10^2, 10^3, 10^4, 10^5, 10^6 and 10^7 cells/mL was prepared. One millilitre of each concentration was

transferred to an Eppendorf tube and centrifuged at 5000 x g for 10 min. The resulting pellets were each resuspended in 100 µL PSS and subsequently submitted to a DNA extraction procedure using the Zymo Fungal/Bacterial kit according to manufacturer instructions. The procedure was repeated for the internal standard, *G. candidum* CBS 109.12. However, in this case the concentration range was prepared with spores, suspended in PSS directly from a plate culture using a sterile inoculating loop.

QPCR reactions

Reactions were performed in 20 µL LightCycler capillaries (Roche Diagnostics, Basel, Switzerland) with the addition of 12.5 µL Taqman Universal Master Mix (Roche Diagnostics, Basel, Switzerland), a 5 µL mixture of forward and reverse primers (5 µM each; Inqaba Biotechnical Industries, Pretoria, RSA) with 400 nM Taqman probe (3' FAM labelled and 5' TAMRA quencher labelled; Inqaba Biotechnical Industries, Pretoria, RSA), 2.5 µL bovine serum albumin (2 mg/mL; Roche Diagnostics, Basel, Switzerland) and 5 µL DNA template. Thermal cycling was initiated with 2 min at 50 ℃ and 10 min at 95 ℃, followed by 40 cycles of 15 s at 95 ℃ and 1 min at 60 ℃. A final cooling stage of 30 s at 40 ℃ completed the programme. Both primers and the probe were designed by Brinkman *et al.* (2003), with the following sequences:

Calb F1 (5'-CTTGGTATTTTGCATGTTGCTCTC-3'),
Calb R1 (5'-GTCAGAGGCTATAACACACAGCAG-3')
Calb P1 (5'-TTTACCGGGCCAGCATCGGTTT-5')

The $\Delta\Delta C_T$ comparative cycle threshold method was used to determine the quantities of target cells, as described in Brinkman *et al.* (2003). This involves comparing the relative quantities of the target sequence from an unknown environmental sample and the quantity of target sequences extracted from a calibrator sample, prepared with a defined concentration of the target sequence, to a standard curve with a known range of target sequence concentrations. Normalising for the relative quantities of recovered DNA in the extraction process from the two different samples and the standard samples was achieved by comparing the recovered target sequence concentration of an external reference strain added in equal quantities to all samples. *Geotrichum candidum* CBS 109.12 was chosen as the reference strain. The comparative cycle thresholds and amplification efficiency of the target assay were calculated as follows, using the Roche LightCycler Software, Version 4.05 (Roche Diagnostics, Basel, Switzerland):

$$\Delta C_{T\,(Test)} = C_{T\,(Target)} - C_{T\,(Ref)} \quad \text{(Eq. 1)}$$

Where $\Delta C_{T\,(Test)}$ referred to the unknown test sample, $C_{T\,(Target)}$ was the cycle threshold of the target sequence (*C. albicans*) in the unknown test sample and $C_{T\,(Ref)}$ was the cycle threshold of the reference sequence (*G. candidum* CBS 109.12) in the same unknown test sample. The same applied to the calibrator sample and all the standard samples.

$$\Delta\Delta C_T = \Delta C_{T\,(Test)} - \Delta C_{T\,(Calibrator)} \quad \text{(Eq. 2)}$$

Where $\Delta\Delta C_T$ referred again to the target sequence (*C. albicans*) in the unknown test sample, $\Delta C_{T\,(Test)}$ referred to the target sequence (*C. albicans*) in the unknown sample normalised with the reference sequence (Eq. 1) and $\Delta C_{T\,(Calibrator)}$ referred to the target sequence (*C. albicans* CAB 628-1) in the calibrator sample normalised with the reference sequence (Eq. 1).

$E^{-\Delta\Delta CT}$ described the ratio of the target sequences in the test and calibrator samples, with E representing the amplification efficiency of the target assay. Estimating the quantities of target cells in a sample involved multiplying this ratio with the known number of target organisms in the calibrator sample. Final calculated concentrations were divided by 100, to compensate for concentrating 100 mL aliquots into 1 mL samples, analysed by the same procedure as the 1 mL standard samples.

Final *C. albicans* concentrations in each sample were used to quantitatively compare the presence of the yeast in the various river zones (W, R and P) as the seasons progressed and coliform and total yeast counts fluctuated in the rivers.

2.1.2 Strains, Sources and Maintenance

2.1.2.1 Sources and maintenance

Candida albicans CAB 628-1 and *C. albicans* CAB 628-2 were obtained from the culture collection of the Department of Microbiology, University of Stellenbosch, South Africa and both originated from domestic livestock. The clinical strains, *C. albicans* TH 8908 and *C. albicans* TH 8912, were sourced from Tygerberg Hospital, Cape Town, South Africa. All strains were maintained on YM agar at 30 ℃. Typical characteristics of *C. albicans* include chlamydospore formation under anaerobic conditions

(Nobile *et al.*, 2003) and hyphal formation upon inoculation in egg white (Buckley and van Uden, 1963), and both of these characteristics were periodically evaluated in all four strains to confirm that the strains did not develop atypical characteristics during maintenance. In addition, these characteristics were also periodically confirmed in randomly selected *C. albicans* strains isolated from the rivers. Chlamydospore formation was demonstrated under semi-anaerobic conditions by the Dalmau inoculation technique, according to Nobile *et al*. (2003). *Candida albicans,* diluted to a final concentration of 10^3 cells/mL in PSS, was spotted onto cornmeal agar [17 g/L cornmeal agar (Difco, Michigan, USA)] covered with a sterile glass coverslip and incubated at 26 ℃ for 7 days in the dark. The plates were subsequently examined for the presence of spores under 15 X magnification on a Nikon SMZ-10A stereoscopic microscope (Nikon, Tokyo, Japan). Hyphal formation was confirmed by *C. albicans* inoculation in 0.5 mL egg white under non-sterile conditions according to the method of Buckley and Van Uden (1963). This method was modified by adding a 100 µL liquid inoculum (diluted in PSS to a final concentration of 10^3 cells/mL) per tube. The tubes were incubated at 37 ℃ and 10 µL samples were examined at 1000 X magnification (Nikon Eclipse E200; Nikon, Tokyo, Japan) every half an hour for evidence of hyphal development. Microscopic imaging took place with a Nikon Coolpix E990 digital camera (Nikon, Tokyo, Japan). The identities of the strains were periodically confirmed as *C. albicans* by transfer to a commercial differential medium for *Candida* species identification, Candiselect4 (as described in 2.1.1.3). This identity confirmation took place before and after each experiment, approximately once a week. In addition, laboratory strain identities were confirmed four times (at approximately six-month intervals) with yeast DNA extraction, followed by molecular analysis of the taxonomically relevant D1/D2 and internal transcribed (ITS) gene regions, as described by Vreulink *et al*. (2010).

2.1.2.2 Molecular identification of yeast isolates

Genomic DNA extraction

Genomic DNA was isolated from yeasts in log phase, sampled after incubation on a rotary shaker (150 rpm, 25 mm throw) in 30 mL YM broth in 250 mL conical flasks at 30 °C. All chemicals were obtained from Merck (Darmstadt, Germany) unless otherwise indicated. Two millilitres of each yeast culture was collected in 2 mL Eppendorf tubes and centrifuged (13 000 x g, 5 min). After resuspension of the pellet in 500 µL DNA lysis buffer containing 20 % (v/v) 1 M Tris-HCl (pH 8.0), 10 % (v/v) 0.5 M EDTA, 5 % (v/v) 20 % (w/v) SDS in sterile distilled water; the samples were vortexed (maximum speed) with acid-washed glass beads and cooled on ice for 5 min. Each sample was mixed

with 275 µL ammonium acetate (pH 7.0), incubated at 65 °C for 5 min and subsequently cooled on ice for 5 min. Following the addition of 500 µL chloroform, the samples were centrifuged (13000 x g, 5 min, 4 ℃) and the supernatant precipitated with 1 volume isopropanol at room temperature for 5 min. The samples were centrifuged (13000 x g, 5 min, 4 ℃), each pellet washed with 1 mL 70 % (v/v) ethanol and again centrifuged (13000 x g, 5 min, 4 ℃). Pellets were dried, dissolved in 50 µL TE buffer and incubated at 37 °C for 20 min. The purified, buffered DNA was stored at -20 °C for further analysis.

PCR amplification of taxonomically relevant gene sequences

Amplification of the D1/D2 region of the 26S ribosomal RNA gene, for molecular identification of the yeasts, was performed using the KAPATaq Ready Mix containing DNA polymerase (KAPA Biosystems, Cape Town, RSA). A modification of the standard protocol was followed, with the addition of 10 µL Ready Mix, 0.8 µL per primer (Inqaba Biotechnical Industries, Pretoria, RSA), 1 µL DNA and 7.4 µL ddH_20. An initial denaturation at 95 °C for 7 min was followed by 30 cycles, each with a denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s followed by an elongation step at 72 °C for 1 min, carried out in an Applied Biosystems 2720 Thermal Cyler (California, USA; Fell *et al.,* 2000). The final elongation step was carried out at 72 °C for 2 min and PCR products were visualised under UV light (Geneflash Syngene Bioimaging Unit, Cambridge, UK) after electrophoresis in a 0.8 % (w/v) agarose gel (Horizon 11.4 GIBCO DRL Horizontal Gel Electrophoresis Apparatus, Life Technologies, California, USA). The PCR products were purified with Nucleospin® Extract II chromatography columns (Macherey-Nagel, Duren, Germany). Samples were sequenced with an ABI PRISM (model 3100) genetic sequencer (Applied Biosystems, California, USA) and the forward and reverse sequences were aligned and edited with DNAMAN for WINDOWS Version 4.13 (Lynnon Biosoft). The online Basic Local Alignment Search Tool (BLAST) was used to detect homology with known species on the NCBI (National Centre for Biotechnology Information) database (http://www.ncbi.nlm.nih.gov/BLAST/).

The D1/D2 gene region was amplified using the following primers:

F63 (5'-GCATATACAATAAGCGGAGGAAAAG-3')

LR3 (5'-GGTCCGTGTTTCAAGACGG-3')

Amplification of the ITS gene region followed the same procedure, amended with the following primers also obtained from Inqaba Biotechnical Industries (Pretoria, RSA), as described by White and co-workers (1990).

ITS1 (5'-TCCGTAGGTGAACCTGCGG-3')

ITS4 (5'-TCCTCCGCTTATTGATATGC-3')

2.1.3 *Candida albicans* Survival in the Rhizosphere

2.1.3.1 Wetland flora

Representatives of three wetland plant genera common to the Western Cape, South Africa, were removed from a natural wetland site (33°49'05"S, 18°41'12"E) surrounded by a residential area, upstream from any stormwater drainages into the river. The plants belonged to the genera *Hydrilla*, *Scirpus* and *Typha* (Fig 1). *Typha*, generally referred to as bulrush, is a robust, reed-like plant common in South Africa (Shode *et al*., 2002). The genus *Scirpus* also consists of bulrush-like emergent macrophytes, which usually occupy the outer zone of estuaries, nearest to open water (Clevering, 1994). They are smaller and more grass-like than *Typha*. The submerged macrophyte, *Hydrilla*, is considered an invasive aquatic weed (Albrecht *et al*., 2004). It is native to Australia, but nevertheless a widespread invasive problem. Two *Typha* plant groups, flowering and non-flowering, were evaluated. Each *Typha* (*ca.* 1 m high) and *Scirpus* (*ca.* 50 cm high) plant was transplanted into 3.5 kg (in 5 L plastic containers) and 2.5 kg (in 3 L plastic containers) of soil respectively. This was conducted in duplicate, one set in garden soil and one set in wetland mud obtained from the same site as the plants. Each *Hydrilla* (*ca.* 25-50 cm long) plant was submerged in 1 L tap water within a 2 L plastic container. Except for the four *Hydrilla* plants, four plants of each group (flowering *Typha*, non-flowering *Typha* and *Scirpus*) were thus cultivated in garden soil and in mud, both saturated with tap water. All 28 microcosms were maintained in a glasshouse with an average day/night temperature and humidity of 23/15 °C and 35/75 % respectively. The above-mentioned flora experiments were conducted under non-sterile conditions, after allowing the plants to adjust for three months to the glasshouse environment.

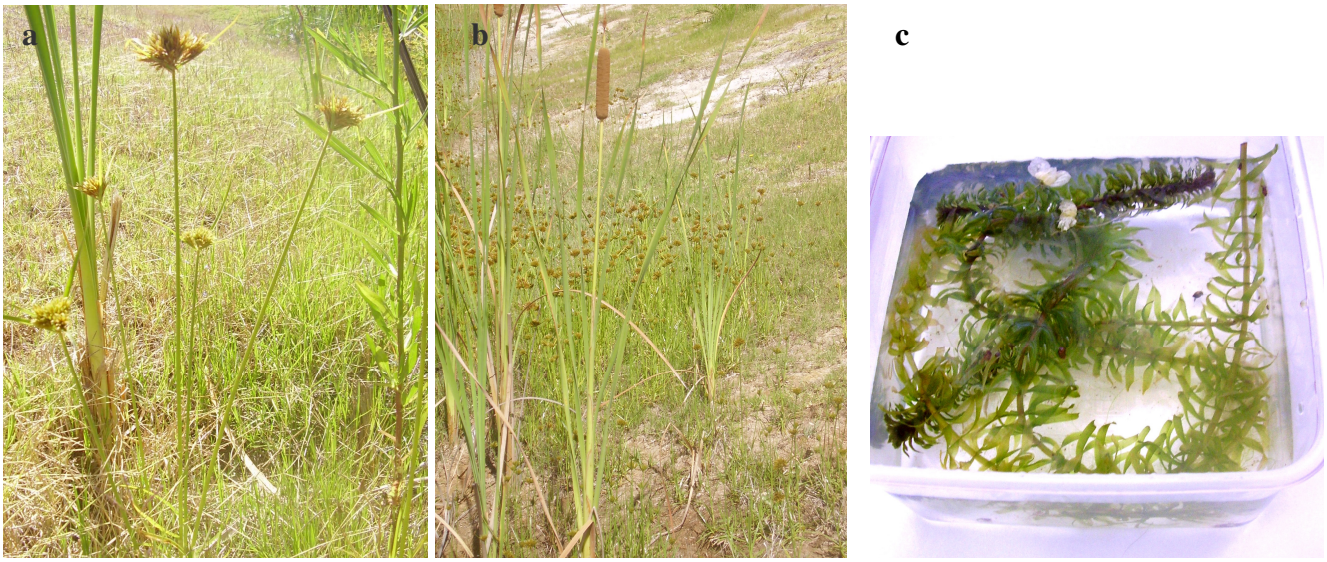

Figure 1. *Candida albicans* survival was studied in the rhizosphere of common wetland macrophytes (images taken at the site described in 2.1.3.1). These macrophytes included (a) *Scirpus*, (b) *Typha* and (c) submerged *Hydrilla* species; obtained from a wetland region in Cape Town, South Africa.

Yeast inocula were prepared by culturing *C. albicans* CAB 628-1 at 30 ℃ in 250 mL conical flasks containing 30 mL YM broth on a rotary shaker (150 rpm, 25 mm throw) in the dark. The yeast cells, allowed to grow to log phase, were subsequently harvested via centrifugation at room temperature in 2 mL Eppendorf tubes (5000 x g, 10 min). The resulting pellet was washed thrice with PSS and finally resuspended in PSS. Yeast concentrations were determined microscopically using direct cell counts on a haemocytometer, and diluted to the correct concentration in PSS. Three plants per genus per soil type were then inoculated with *C. albicans* CAB 628-1, resulting in a final concentration of 500 cells/g dry weight of soil (*Typha* and *Scirpus*) or 500 cells/mL water (*Hydrilla*). This yeast inoculum concentration was chosen based upon *C. albicans* numbers commonly reported in wastewater (Cook and Schlitzer, 1981; El-Taweel and Shaban, 2001; Hagler, 1981). Inoculation involved thoroughly mixing the yeast into the soil/water before transplanting the plants into the inoculated soil/water. One uninoculated control was maintained for each experimental group.

Following one month of cultivation, 1 g roots and 1 g bulk soil away from the root (*Scirpus* and *Typha*), and 1 g plant matter and 1 mL bulk water away from the plant (*Hydrilla*), were harvested, added to separate 9.0 mL PSS test tubes and thoroughly vortexed for 20 s. This was carried out in triplicate for each microcosm. Dilution series were prepared, spread plated onto YM agar, and the plates incubated at 37 ℃ for 24 h, whereafter yeast numbers were recorded. The dry weight of all roots and bulk soil samples was determined after drying at 80 ℃ for 24-48 h. Final yeast concentrations

were calculated as cells/g dry weight (*Scirpus* and *Typha*) or cells/g dry weight and cell/mL (*Hydrilla* plants and bulk water respectively). Yeast numbers in the different zones were compared for significant variation, using a Box & Whisker Plot for all variables (Statistica Version 9.0; StatSoft). Yeasts were randomly selected from the YM plates and identities were confirmed as *C. albicans* by incubation on Candiselect4 at 30 ℃ for 24 h, along with molecular identification (2.1.2.2) of two colonies per group.

2.1.3.2 Crop flora

Two common crop plant species, maize (*Zea mays*) and tomato (*Solanum lycopersicum*), were germinated on water agar in direct sunlight at *ca.* 21 ℃, and each plant was subsequently grown in 200 g silica sand (250 – 355 µm grain size; Consol Glass Group, Bellville, South Africa) under non-sterile conditions. Once the plants were approximately 10 cm high, six experimental plants per species were inoculated and maintained in a glasshouse as described above (2.1.3.1), along with three uninoculated controls per species. A notable variation in plant cultivation in comparison to the wetland flora involved watering with 100 mL tap water once a week, rather than maintaining a saturated water capacity. After two weeks, harvesting followed by data gathering and analysis took place as described above (2.1.3.1).

The experiment was repeated in duplicate in order to evaluate the effect of temperature on *C. albicans* survival in the rhizosphere. Wheat (*Triticum aestivum*) seedlings (instead of tomato and maize) were germinated, planted and inoculated with *C. albicans* CAB 628-1 as described above. The wheat was planted in 100 g silica sand (250 – 355 µm grain size) and was watered with 50 mL tap water once a week. The plants were cultivated in separate glass tanks, one set was maintained at 24 ℃ and the other set at 30 ℃, with all other parameters constant. Harvesting and data analyses were performed as described above (2.1.3.1).

2.1.3.3 Microscopic visualisation of rhizosphere effect on *C. albicans*

Wheat (*Triticum aestivum*) seedlings, one per plate, were germinated on water agar (pH 6.0) in which *C. albicans* CAB 628-1 had been suspended at a cell concentration of 200 cells/mL (inocula were prepared as described above, 2.1.3.1). Twenty plates were inoculated with *C. albicans*: ten with germinating seedlings, and ten with ‘dead’ seedlings which had been autoclaved prior to inoculation, at 121 ℃ and 2 kg/cm^2 for 15 minutes. Ten more plates functioned as uninoculated controls, containing

germinating seedlings without *C. albicans*. All were incubated at *ca.* 21 ℃ and exposed to a natural day/night light cycle. After a five day germination period, the plates were examined at 15 X magnification using a Nikon SMZ-10A stereoscopic microscope and imaging took place using a Nikon Coolpix E990 camera. Yeast densities and spatial distribution were visually evaluated in the vicinity of the roots. The experiment was repeated with the other three strains of *C. albicans* (CAB 628-2, TH 8908, TH 8912).

2.1.4 *Candida albicans* Survival on Plant Debris

2.1.4.1 Terminally evaluated survival studies

Twenty (Exp. A, Table 1) and subsequently 32 (Exp. B, Table 2) microcosms were set up, representing, respectively, five and eight different treatments in which the survival of *C. albicans* CAB 628-1 was evaluated. Three identical experimental microcosms and one uninoculated control microcosm were prepared for each treatment. The treatments and microcosm compositions are summarised in Tables 1 and 2.

Table 1. Microcosm contents and treatment conditions for the survival study of *C. albicans* CAB 628-1 in various soil types, Experiment A.

Substrate	Consortium	Oxygen Exposure	Amount (g)	H_20 (mL)
Straw	Non-sterile	Anaerobic	2.0	20.0
Sand			10.0	20.0
Mud			25.0	20.0
Wetland Mud + Plant Debris (Mixed)			25.0 + 2.0	20.0
Wetland Mud + Plant Debris (Unmixed)			25.0 + 2.0	20.0

Table 2. Microcosm contents and treatment conditions for the survival study of *C. albicans* CAB 628-1 on plant debris, Experiment B.

Substrate	Consortium	Oxygen Exposure	Amount (g)	H_20 (mL)
Straw	Sterile	Aerobic	2.0	20.0
		Anaerobic	2.0	20.0
	Non-sterile	Aerobic	2.0	20.0
		Anaerobic	2.0	20.0
Wetland Plant Debris	Sterile	Aerobic	3.0	20.0
		Anaerobic	3.0	20.0
	Non-sterile	Aerobic	3.0	20.0
		Anaerobic	3.0	20.0

Microcosms were set up in 50 mL glass jars, all of which were autoclaved before the contents were added. Shredded wheat straw, included as a substrate in selected microcosms, was donated by a nearby farm, whilst wetland plant debris (including the roots, stems and leaves of dead *Scirpus* and *Typha*) and mud were obtained from the same wetland site reported in 2.1.3.1. As previously mentioned, the sand (250 – 355 µm grain size) was obtained from Consol Glass Group. Plant debris was assessed as 'dead' by a completely brown colour. No green plant matter was harvested for the microcosms. Experimental microcosms were inoculated with *C. albicans* CAB 628-1, as described in 2.1.3.1, to a final concentration of 500 cells/g dry weight. Anaerobic conditions were maintained by incubating selected microcosms in anaerobic jars (Merck, Darmstadt, Germany) with Microbiology Anaerocult A packs (Merck, Darmstadt, Germany). Anaerobic conditions were monitored with Microbiology AnaeroTest strips (Merck, Darmstadt, Germany). Exp. B substrate sterility was obtained by radiation, thus eight straw microcosms and eight wetland plant debris microcosms were exposed to a radiation dose of 20.0 kGy (Hepro, Cape Town, RSA). For Exp. A, Wetland Mud + Plant Debris (Mixed) refers to microcosms in which the inoculum, mud and plant debris were thoroughly mixed, and initial and final yeast concentrations were determined per g dry weight of the mixture. Wetland Mud + Plant Debris (Unmixed) refers to microcosms in which the inoculum and plant debris were mixed and incubated on top of the mud zone (similar to wetland environments), and initial and final yeast concentrations were calculated per g dry weight of the plant debris. All microcosms were incubated at 26 ℃ in the dark for 15 days. After 15 days, the microcosms were thoroughly mixed, 1 g of substrate per microcosm was sampled in triplicate and vortexed for 20 s in separate 9.0 mL PSS test tubes. Dilution series were plated out on YM agar and analysed as described in 2.1.3.1. On a separate occasion treatments

involving wetland plant debris (Table 2) were repeated using inocula prepared with *C. albicans* CAB 628-2, *C. albicans* TH 8908 and *C. albicans* TH 8912, to confirm that the response was not strain-specific.

Similar microcosms were set up following an analogous procedure with wetland mud (Table 1), subject to three treatments: non-sterile, autoclave sterilised and sterilised with radiation (20.0 kGy). Each microcosm contained 25 g wetland mud. The experiment was performed in duplicate, using *C. albicans* CAB 628-2 and *C. albicans* TH 8912. Harvesting, data gathering and analysis took place after two weeks as described above.

2.1.4.2 Growth of *C. albicans* on wetland plant debris monitored over one month

Microcosms containing sterile or non-sterile plant debris were set up and inoculated with *C. albicans* CAB 628-1 as described above (2.1.3.1 and 2.1.4.1), with treatment differences highlighted in Table 3. Twenty microcosms were sterilised by radiation (20.0 kGy). Twenty were left unsterilised. Ten sterile and ten non-sterile microcosms were incubated anaerobically, whilst the rest were incubated aerobically at 26 ℃ in the dark. Of the ten microcosms per treatment, eight inoculated microcosms and two uninoculated controls were prepared.

Table 3. Microcosm contents and treatment conditions for the growth curve of *C. albicans* CAB 628-1 on wetland plant debris.

Substrate	Consortium	Oxygen Exposure	Amount (g)	H_20 (mL)
Wetland Plant Debris	Sterile	Aerobic	7.0	20.0
		Anaerobic	7.0	20.0
	Non-sterile	Aerobic	7.0	20.0
		Anaerobic	7.0	20.0

For a period of 30 days, every six days the microcosms were thoroughly mixed, 1 g of plant matter/microcosm was sampled and dilution series were plated out and analysed as described in 2.1.3.1. It must be noted that due to the gradual removal of oxygen by the Microbiology Anaerocult A packs in anaerobic jars, the anaerobic microcosms were exposed to gradually decreasing oxygen levels for approximately two hours every six days after sampling. However, measures were taken to ensure minimal oxygen exposure, including spreading the microcosms in several anaerobic jars, tightly sealing

all microcosms, opening jar by jar and microcosm by microcosm, and immediately placing the microcosms back in the anaerobic jars after sampling.

2.1.5 Elucidation of *C. albicans* Growth Substrates in Wetland Plant Debris

2.1.5.1 Aerobic and anaerobic acid production by *C. albicans*

All four *C. albicans* strains (CAB 628-1, CAB 628-2, TH 8908, TH 8912) were inoculated in triplicate onto differential agar plates containing a sole carbon source, yeast nitrogen base [0.67 % (w/v) filter-sterilised YNB; Difco, Michigan, USA] and 0.002 % (w/v) bromophenol blue (Merck, Darmstadt, Germany). The carbon sources were 1 % (w/v) glucose; 1 % (w/v) cellobiose and 1 % (w/v) carboxymethylcellulose (CMC), all obtained from Sigma Aldrich (Missouri, USA). Cultures were prepared for inoculation as described in 2.1.3.1. Subsequently, two triplicate sets of streak cultures of each strain were prepared on the differential media containing different carbohydrates as sole carbon source. The resulting plate cultures were incubated at 26 ℃ in the dark (Powell, 1995); one set incubated aerobically, whilst the other was incubated anaerobically using Anaerocult A packs in anaerobic jars. The plates were monitored daily for colour variations, and imaged (Olympus D-535 digital camera; Olympus, Tokyo, Japan) once acidification was evidenced by a colour conversion of the medium from blue to yellow. Plates incubated aerobically with *S. cerevisiae* ABO-SC1 served as a negative control.

2.1.5.2 Aerobic and anaerobic cellulase activity of *C. albicans*

All four *C. albicans* strains (CAB 628-1, CAB 628-2, TH 8908, TH 8912) were inoculated in triplicate onto 1 % (w/v) CMC agar plates supplemented with 0.67 % (w/v) YNB and incubated for three days at 30 ℃ in the dark (Botes *et al*., 2009). Cultures were prepared for inoculation as described in 2.1.3.1. However, subsequent to growth in liquid YM, the yeast was transferred to a 250 mL conical flask containing 30 mL of a 0.1 % (w/v) glucose solution in 0.67 % (w/v) YNB, and cultivated for 24 h on a rotary shaker (150 rpm, 25 mm throw) at 30 ℃. This was carried out in order to deplete the sugar reserves of the yeast before streaking out on the CMC-containing plates, according to Wickerham and Burton (1948). Two triplicate sets were inoculated; one incubated aerobically, the other anaerobically with Anaerocult A packs in anaerobic jars. Following the incubation period, the plates were stained for 15 minutes with a 0.1 % (w/v) congo red solution (B&M Scientific, RSA) and destained for 15 minutes

with a 1 M NaCl solution. Plates inoculated with *S. cerevisiae* ABO-SC1 served as a negative control, whilst plates inoculated with *Trichoderma reesei* ABO-TR1 served as a positive control. Clear zones surrounding the colonies represented cellulase activity, and all plates were imaged (Olympus D-535 digital camera). The experiment was repeated adding 0.1 % (w/v) glucose, sucrose and xylose separately to CMC plates, and analysed as described above in order to evaluate whether the presence of simple mono- and disaccharides could stimulate the utilisation of cellulose (Pearson *et al*., 1990). Once again, all carbohydrates were obtained from Sigma Aldrich (Missouri, USA).

2.1.5.3 Aerobic and anaerobic cellobiose utilisation of *C. albicans*

The *C. albicans* strains (CAB 628-1, CAB 628-2, TH 8908, TH 8912) were also analysed for cellobiose assimilation. Test tubes containing 9.9 mL 0.67 % (w/v) YNB and 5 % (w/v) cellobiose were inoculated in triplicate with 100 µL of a 10^6 cells/mL *C. albicans* suspension (cultured as described in 2.1.3.1). The inocula were also grown under carbon limitation as described in 2.1.5.2. Two sets of triplicate tubes were prepared; one for aerobic incubation, the other for anaerobic incubation. Three uninoculated tubes served as negative controls, whilst three tubes inoculated with *T. reesei* ABO-TR1 served as positive controls. Both aerobic and anaerobic (maintained with Anaerocult A packs in anaerobic jars) cultures were incubated on a rotary shaker (150 rpm, 25 mm throw) at 26 ℃ in the dark for 3 days. The absorbance (SmartSpec Plus, Bio-Rad, California, USA) at 600 nm was determined for each tube and positive growth evaluated as OD_{600} values significantly greater than those of the uninoculated negative controls. The positive controls were visually evaluated, with fungal hyphae demonstrating positive growth. The experiment was repeated on randomly selected *C. albicans* strains isolated from the Plankenburg and Diep Rivers during this study.

2.1.5.4 Aerobic and anaerobic fibre degradation capabilities of *C. albicans*

ANKOM technology was used to analyse the aerobic and anaerobic insoluble fibre degradation capabilities of *C. albicans* with wheat straw as substrate. Specifically, the neutral detergent (ND) filter bag method was employed, as initially described by Van Soest *et al*. (1991) and Vogel *et al*. (1999). All straw samples were prepared by milling, sifting (100 µm pore size) and overnight drying at 80 ℃ to determine pre-analysis dry weights. Filter bags (Ankom F57 filter bags, Ankom Technology Corp., Fairport, USA) were pre-rinsed in acetone (Saarchem, Krugersdorp, RSA) to remove microbial inhibitors, air-dried, marked, oven-dried at 100 ℃ overnight and placed in a dessicator for 3 h.

After dessication, all filter bags were weighed, 0.5 g (±0.01 g) processed straw samples were added to each bag and each bag was heat-sealed. The reference bags were weighed and sealed with no substrate added. The bags were subsequently sterilised by radiation (20.0 kGy) and added to 100 mL sterile basal medium (pH 5.0, Table 4) supplemented with chloramphenicol (600 mg/L) in 100 mL Schott bottles (1 bag/ bottle). The medium was kept as anaerobic as possible by avoiding agitation. The bottles were inoculated with a yeast suspension (grown under carbon limitation, as described in 2.1.3.1 and 2.1.5.2) resulting in a final concentration of 10^6 cells/mL. The cultures were then tightly sealed and incubated at 30 ℃ in the dark. Yeasts were kept in suspension with a Variomag Poly 15 electromagnetic stirrer (Munich, Germany). Each strain was inoculated in triplicate, and an uninoculated group was added in triplicate as a negative control, in addition to the reference bags without substrate. The *C. albicans* strains analysed included CAB 628-1, CAB 628-2, TH 8908 and TH 8912. To test for aerobic fibre degradation, the experiment was repeated in tandem using identical procedures, except that the Schott bottles were replaced by 500 mL conical flasks with cotton wool plugs.

Table 4. Composition of the ANKOM basal/minimal medium.

Components[1]	**Weight (g/L)**
Tween80	1.000
KNO_3	6.000
KH_2PO_4	12.00
$(NH_4)_2SO_4$	5.000
KCl	1.600
$MgSO_4.7H_2O$	1.200
$ZnSO_4.7H_2O$	0.010
$MnSO_4.6H_2O$	0.009
$CuSO_4.7H_2O$	0.009
Urea	2.000

[1]All chemicals were obtained from Merck (Darmstadt, Germany).

After an incubation period of one week, all bags were removed from the cultures, dried at 100 ℃ for 24 h, weighed and subjected to the NDF analysis method, as described by Van Soest *et al.*, 1991 and Vogel *et al.*, 1999. The bags were suspended (three per tray) in 2 L Neutral Detergent Solution comprising sodium lauryl sulphate, 3 % (w/v); EDTA, 1.861 % (w/v); sodium tetraborate decahydrate, 0.681 % (w/v); sodium phosphate dibasic anhydrous, 0.451 % (w/v) and triethylene glycol, 1 % (v/v) in

deionised water (pH 7.0 ±0.1). The media was simultaneously agitated and heated, and subsequently supplemented with 0.1 % (w/v) sodium sulphite (Sigma Aldrich, Missouri, USA). An ANKOM Fibre Analyser 200/220 (ANKOM Technology, Fairport, USA) was used for processing at 99 ℃ for 75 min. Two 5 min rinses with 90 ℃ tap water supplemented with 0.2 % (v/v) heat stable alpha-amylase (ANKOM Technology, Fairport, USA) were followed by a final rinse step without the enzyme. The bags were subsequently submerged in acetone for 5 min, air-dried completely, oven-dried at 102 ℃ for 4 h and finally cooled in a dessicator.

After weighing, the % NDF (Neutral Detergent Fibre) was calculated as:
[(Post-digestion sample weight including filter bag – Bag tare weight x Blank bag correction)/Sample weight] x 100 (Eq. 3)
Blank bag correction = Final oven-dried reference bag weight/original reference bag weight

Significant differences between the percentage degraded NDF (uninoculated and inoculated) were evaluated with a Box and Whisker plot for all variables, using Statistica Version 9.0 (Statsoft).

2.1.5.5 Aerobic and anaerobic utilisation of free sugars and nitrogen in plant debris by *C. albicans*

Using a Dionex high performance liquid chromatography (HPLC) system, an anionic exchange method with pulsed amperometric detection (HPAE-PAD) was used to preliminarily determine the free mono- and disaccharides utilised by *C. albicans* CAB 628-1 in a sterile wetland plant debris extract. In addition, a commercial analysis evaluated nitrogen utilisation by *C. albicans* in the same sterile wetland plant debris extract. Finally, gas chromatography coupled with mass spectrometry (GC-MS) was subsequently employed for a more detailed analysis of the mono- and disaccharide depletion in wetland plant extract inoculated with *C. albicans.*

Preparation of plant extracts
Plant extracts were prepared by harvesting dead plant debris (brown *Scirpus* and *Typha* leaves, stems and roots) from the wetland site referred to in 2.1.3.1 and cutting the plant matter into approximately 2 mm x 2 mm fragments. Sixty grams of plant matter, submerged in 10 mL distilled water per gram of plant matter, was exposed to 121 ℃ and 2 kg/cm^2 for 15 min in an autoclave, in order to release soluble sugars from the plant biomass. A Whatman 2 filter was used to remove particulate matter from the extract, and the resulting filtrate was subsequently sterilised in a Sartorius polycarbonate filter

system using a cellulose acetate filter disk with a 0.22 µm pore size (Sartorius Stedim Biolab Products, Aubagne, France).

Experimental design

Sterile test tubes each received 9.9 mL of the above-mentioned filter-sterilised plant extract and 0.1 mL of a yeast suspension in PSS, resulting in final concentration of 1 x 10^6 cells/mL. The yeast suspension was prepared by culturing and diluting *C. albicans* CAB 628-1 as described in 2.1.3.1, including an incubation period under carbon limitation in 0.67 % (w/v) YNB supplemented with 0.1 % (w/v) glucose, as described in 2.1.5.2. Three samples were inoculated with the yeast suspension and three controls were prepared by substituting the inoculum with 0.1 mL PSS. All tubes were thoroughly vortexed (maximum speed) and incubated with Anaerocult A packs in anaerobic jars on a rotary shaker (150 rpm, 25 mm throw) at 30 ℃ for 7 h. Growth curves obtained during preliminary experiments, with hourly readings at $OD_{600,}$ indicated that *C. albicans* strains enter log phase after *ca.* 6 h of incubation and stationary phase after *ca.* 8 h of incubation when cultured under these conditions. The yeast cultures were therefore sampled at approximately mid-exponential phase. During sampling, the test tubes to be sampled were thoroughly vortexed and 2 mL of culture fluid per tube was extracted and filter-sterilised with 0.22 µm pore-size syringe filters (GVS Filter Technologies, Bologna, Italy). The sterile filtrate was subsequently transferred to new Eppendorf tubes for further analysis by HPLC, as well as nitrogen profiling.

HPLC sample and standard preparation and analysis

Standards were prepared by diluting 0.100 g of each sugar in 100 mL deionised water, and this stock was used to set up a concentration range of 5 ppm to 100 ppm. Pure sugars included in the standard were D-fructose, D-glucose, D-sucrose, D-xylose and L-arabinose. All samples and reagents were prepared with deionised water (18.2 megaohms-cm resistivity) and reagents were of >99.8 % purity, obtained from Sigma-Aldrich (Missouri, USA). All standards and samples were filter-purified (0.22 µm pore-size syringe filters) immediately prior to analysis.

Samples and standards were analysed with a Dionex Ultimate 3000 series (Dionex Corp., California, USA), equipped with a dual gradient pump, a Carbopac PA1 guard (4 x 50 mm^2) and an analytical column (4 x 280 mm), as well as a Coulchem III electrochemical detector for pulsed amperometric detection. The column was eluted using an isocratic gradient with 30 mM NaOH (1 mL/min) and samples were kept cold during the analysis. The concentrations of individual mono- and disaccharides

were analysed using the Chromeleon Dionex Software, Version 6.80 SR8 (Dionex Corp., California, USA). All standards and samples were injected in 20 µL volumes. Standard specificity, linearity of the detector, precision, accuracy and limits of detection were analysed before and after column cleanings, in order to assess the robustness and accuracy of the system.

Due to the environmental nature of the sample, impurities may have influenced the data and some sugars were present in notably low concentrations. Therefore, in order to verify the data, all sugar quantifications were normalised with the arabinose ratio of the control and experimental samples, since arabinose was present in significant amounts and not assimilated anaerobically by *C. albicans*. Samples were analysed for significant differences in individual sugar concentrations with a Box and Whisker plot for all variables, using Statistica Version 9.0 (Statsoft).

Nitrogen profiling of the plant extract

The total nitrogen profiles were determined for the above-mentioned samples by a commercial laboratory (BEMLab, Somerset West, RSA), in order to compare inoculated and uninoculated samples. Total nitrogen was evaluated by digestion in a LECO FP-528 nitrogen analyser with Spectrascan standards (Drobak, Norway); while the ammonium, nitrite and nitrate content was determined in a 1 mol/L KCl extract (Bremner, 1965).

GC-MS sample and standard preparation and analysis

New plant extracts were prepared and cultured with all four strains of *C. albicans* (CAB 628-1, CAB 628-2, TH 8908, TH 8912), precisely as described above for HPLC (2.1.5.5. Preparation of plant extracts and Experimental design). An Agilent 6890N gas chromatograph (Santa Clara, USA) and an Agilent 5975 mass spectrometer (Santa Clara, USA) were coupled for a GC-MS system analysis. Gas chromatography was performed on a 30 m HP5 column (Santa Clara, USA), with a 0.25 mm inner diameter and a 0.25 µm film thickness. The injection temperature was 260 ℃, the interface set to 280 ℃ and the ion source adjusted to 230 ℃. Helium, the carrier gas, was set at a constant flow rate of 1 mL/min. Sample volumes of 1 µL were injected with a split ratio of 1:10 using a hot-needle technique. The temperature program involved 5 min isothermal heating at 70 ℃, followed by a 1 ℃/min oven temperature ramp to 76 ℃, a second ramp of 5 ℃/min to 310 ℃, and a final hold at 310 ℃ for 8 min. Mass spectra were recorded at two scans per sec with an *m/z* 40-600 mass scanning range. The chromatograms and mass spectra were evaluated using the Enhanced MSD ChemStation Software, Version D.02.00.237 (Agilent Technologies, Santa Clara, USA).

Pure mono- and disaccharides included in the standard were D-fructose, D-galactose, D-glucose, D-sucrose and D-xylose. All samples and reagents were prepared with deionised water (18.2 megaohms-cm resistivity) and all reagents were of >99.8 % purity, obtained from Sigma-Aldrich (Missouri, USA). Each standard ranged from 5 ppm to 100 ppm, and each 2 mL Eppendorf tube contained 200 µL of the standard, in addition to 200 µL of the internal standard, ribitol (100 ppm). A cocktail was prepared containing 100 ppm of each sugar (200 µL), along with 200 µL of ribitol (100 ppm). Samples were prepared by adding 200 µL of plant-filtrate to 200 µL ribitol (100 ppm) in 2 mL Eppendorf tubes. The tubes were subsequently frozen at -80 ℃ for 24 h, and lyophilised using a VirTis 6K BenchTop freeze dryer (VirTis, NY, USA). The dried residue was redissolved and derivatised for 90 min at 30 ℃ (in 80 µL of 20 mg/mL methoxyamine hydrochloride in pyridine) followed by a 30 min treatment at 37 ℃ with 140 µL of MSTFA (N-methyl-N-trimethylsilyl-trifluoroacetamide). Forty microlitres of a retention time standard mixture was added prior to trimethylsilylation. This retention time standard mixture contained 3.7 % (w/v) heptanoic acid, 3.7 % (w/v) nonanoic acid, 3.7 % (w/v) undecanoic acid, 3.7 % (w/v) tridecanoic acid, 3.7 % (w/v) pentadecanoic acid, 7.4 % (w/v) nonadecanoic acid, 7.4 % (w/v) tricosanoic acid, 22.2% (w/v) heptacosanoic acid and 55.5 % (w/v) hentriacontanoic acid dissolved in tetrahydrofuran at 10 mg/mL total concentration. The standard solution for peak identification was also derivatised with 80 µL of a 20 mg/mL methoxyamine hydrochloride in pyridine and 140 µL MSTFA, as described above.

Due to the environmental nature of the sample, impurities may have influenced the data. Therefore, in order to verify the data, all sugar quantifications were normalised with the internal standard, ribitol. Samples were analysed for significant differences with a Box and Whisker plot for all variables, using Statistica Version 9.0 (Statsoft).

Anaerobic growth on single mono- and disaccharides

The ability of *C. albicans* to grow anaerobically on each of the above-mentioned mono- and disaccharides as sole carbon source was investigated. One hundred microlitres of each of the four *C. albicans* strains (CAB 628-1, CAB 628-2, TH 8908, TH 8912) was inoculated in test tubes containing 9.9 mL 0.67 % (w/v) YNB supplemented with 1 % (w/v) D-fructose, D-galactose, D-glucose, D-sucrose, D-xylose or D-arabinose as sole carbon source. Preparation for inoculation (2.1.3.1), and pre-inoculation carbon limitation (2.1.5.2), led to a final inoculum concentration of 1 x 10^6 cells/mL. Growth was evaluated spectrophotometrically, in comparison to negative controls, as described in 2.1.5.3.

2.2 Results and discussion

2.2.1 Discovery of *C. albicans* in Oxygen-Limited, Reducing Zones of Wetlands and Rivers

Candida albicans has been repeatedly confirmed as the most prevalent yeast in sewage effluent and sewage-contaminated waters (Arvanitidou *et al*., 2005; Buck and Bubucis, 1978; Efstratiou *et al*., 1998; El-Taweel and Shaban, 2001; Vogel *et al.*, 2007), due to its natural residence in the mammalian gastrointestinal tract (Kumamoto and Vinces, 2005). *Candida albicans* counts and total yeast counts in polluted waters were shown to correlate with the more traditional coliform counts, all of which are indicative of pollution levels (Efstratiou *et al*., 1998). During a preliminary year-long study (2009) of the notably polluted Plankenburg River, we found no correlation between coliform and total yeast counts in the river; which was sampled at random depths predominantly in the clear, flowing zones. During this preliminary study, yeast species were isolated and identified using Candiselect4 and molecular evaluation of taxonomically relevant gene sequences, as described in the present study (2.1.1.1-2.1.1.3, excluding zone distinction). The predominant *Candida* species isolated from the river was *C. tropicalis*, a known resident of rivers and soil (Kurtzman and Fell, 2000). However, no *C. albicans* was detected in the river throughout the 2009 study, even in the dry season when coliform counts were high, indicative of a high wastewater load and a low pathogen dilution factor. This anomaly turned our attention to an investigation of river microhabitats analogous to that of the gastrointestinal origin of *C. albicans* in sewage and polluted waters (De Hoog *et al.*, 2000). Consequently, we enumerated and compared coliform numbers, total yeast numbers and *C. albicans* numbers in specific zones within the Plankenburg and Diep Rivers.

Thus, coliform counts and total yeast counts were monitored for eight months (June 2010 – January 2011) in three distinct zones of the Plankenburg River, all *ca*. 50 cm below water surface level. The zones included clear, flowing water (W); rock-filtered water (R) and plant-filtered water (P). The redox potential of the P zone was found to be consistently *ca.* 80-100 mV lower than that of the W and R zones. Therefore, the P zone is referred to as the oxygen-limited, reducing zone; whereas the W and R zones are referred to as the aerobic, oxidising zones. As the total yeast and coliform numbers within each of the zones increased in the dry season, it is clear that the dilution factor decreased and considerable pollution was evident as the counts peaked (Fig 2a & b). According to the General Authorisations in terms of the National Water Act of South Africa (Government Notice 1191 in the Government Gazette 20526, October 1999), coliform counts exceeding the General Limit of

1000 cfu/100 mL are deemed dangerous. The Plankenburg River exceeded this level considerably during the dry season (Fig 2a). The Diep River, tested on three occasions (data not shown), displayed consistently lower total coliform and yeast concentrations, only slightly exceeding this limit in the dry season. A significant correlation between total coliform concentrations and total yeast concentrations was demonstrated over the sampling period in each of the three zones of the Plankenburg River, (Fig 3). This indicated that these correlations are zone-specific in the W, R and P zones investigated in this study.

Each of the zones (W, R and P) was also evaluated monthly for the presence of *C. albicans* using a semi-quantitative method (Table 5). This method involved filtering a 100 mL water sub-sample from each zone, incubating the filter on SGA at 37 ℃, and randomly selecting colonies from the filter to be identified using Candiselect4. Molecular identification was periodically employed to confirm *C. albicans* identification on Candiselect4. Each of these confirmations was positive, and this may be the first report of the robustness of Candiselect4 for the identification of *C. albicans* outside of the clinical environment. Using this method, the presence of *C. albicans* was demonstrated in the R and P zones early in the rainy season (June 2010 – August 2010) and well into the dry season (Jan 2010), when total yeast and coliform numbers were high indicating considerable pollution in the river (Table 5; Fig 2 & 4). However, as the rainy season progressed and total yeast and coliform (pollution) levels became negligible in all zones of the river, the presence of *C. albicans* remained constant in the P zone, whilst dropping to zero in the R zone (Table 5; Fig 2 & 4). Throughout the monitoring period, no *C. albicans* was found in the W zone (Table 5; Fig 4). The Diep River was also analysed for total yeast and coliform counts, as well as semi-quantitatively for the presence of *C. albicans*, on three separate occasions. Although total yeast and coliform counts were consistently lower than in the Plankenburg River (data not shown), *C. albicans* was shown to be constantly present in the P zone and absent in the W and R zones (Table 5; Fig 5), confirming the survey results of the Plankenburg River.

These semi-quantitative results were confirmed using qRT-PCR (Brinkman *et al*., 2003). *Candida albicans* levels were quantified in each zone three times (Table 6), twice during low pollution levels and once during the dry season, when total yeast and coliform counts were considerable (Fig 2 & 6). *Candida albicans* was again found to be consistently absent in the W zone, whereas the R zone demonstrated approximately 0 cells/100 mL in the rainy season (Table 6, Fig 6) and 10^2 cells/100 mL during the dry season (Table 6). Importantly, *C. albicans* levels remained at 10^2-10^3 cells/100 mL in the P zone throughout the study (Table 6, Fig 6).

The P zone was established as a reducing environment, rich in plant debris; in contrast to the R and W zones which are aerobic, with a higher redox potential and lacking in decaying plant matter. Zones similar to the P zone are typical in wetland and river bank environments, and are characteristically similar to the gastrointestinal tract, the host reservoir of sewage-borne *C. albicans* (Hentges, 1993; Nelson *et al*., 2003; Seybold *et al*., 2002). In our study, as pollution levels decreased in the river, *C. albicans* levels remained constant in this P zone, suggesting the potential of such a wetland/river bank niche for *C. albicans* outside of its human host.

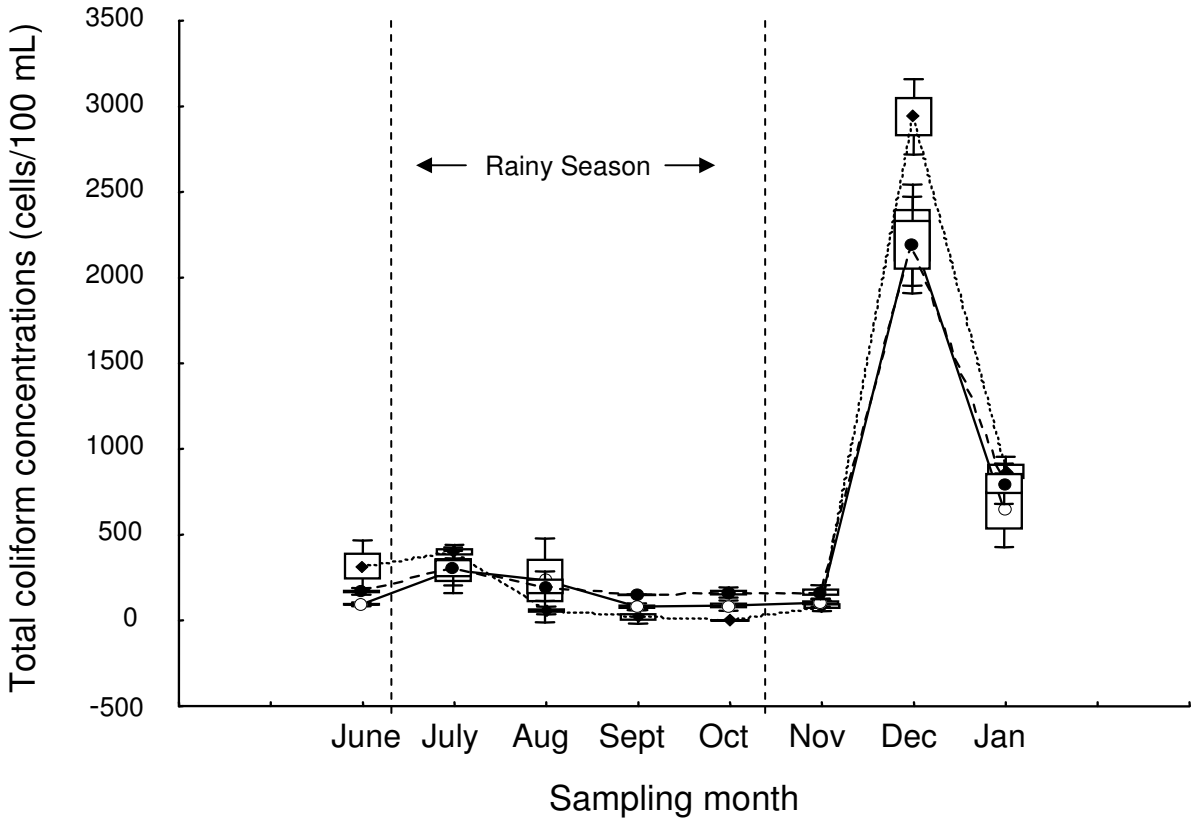

Figure 2a. Total coliform counts in different zones of the Plankenburg River monitored over rainy and dry seasons. The rainy season is indicated, the rest of the year is predominantly dry. The dashed line represents clear, flowing water (W); the solid line represents rock-filtered zones (R) and the dotted line represents plant debris-filtered zones (P). All zones are *ca.* 50 cm below water surface level. Total coliform numbers were determined with three replicates per sample (n=3). Coliform numbers in the respective zones were compared with a Box and Whisker Plot showing mean (♦), mean ± standard deviation (□) and mean ± (1.96 x standard deviation) (⌶).

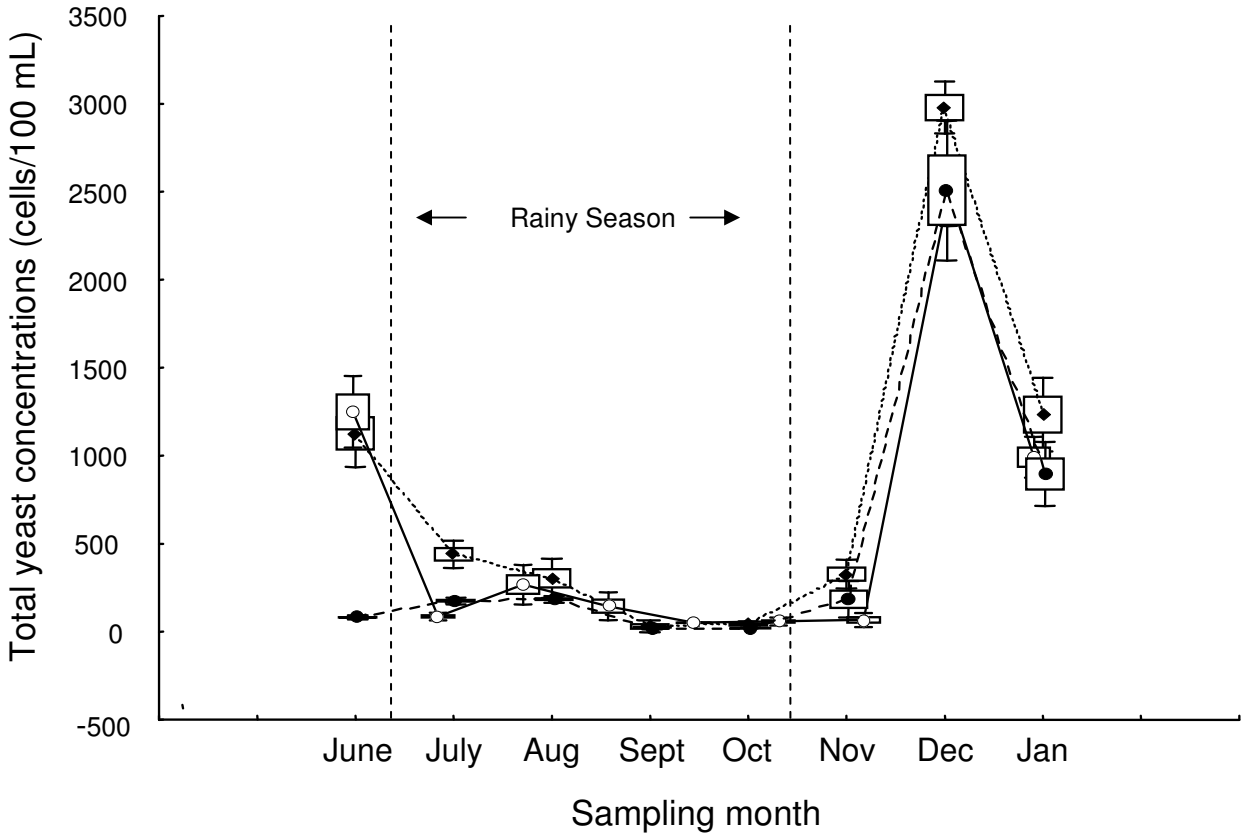

Figure 2b. Total yeast counts in different zones of the Plankenburg River monitored over rainy and dry seasons. The rainy season is indicated, the rest of the year is predominantly dry. The dashed line represents clear, flowing water (W); the solid line represents rock-filtered zones (R) and the dotted line represents plant debris-filtered zones (P). All zones are *ca*. 50 cm below water surface level. Total yeast numbers were determined with three replicates per sample (n=3). Yeast numbers in the respective zones were compared with a Box and Whisker Plot showing mean (♦), mean ± standard deviation (□) and mean ± (1.96 x standard deviation) (⌶).

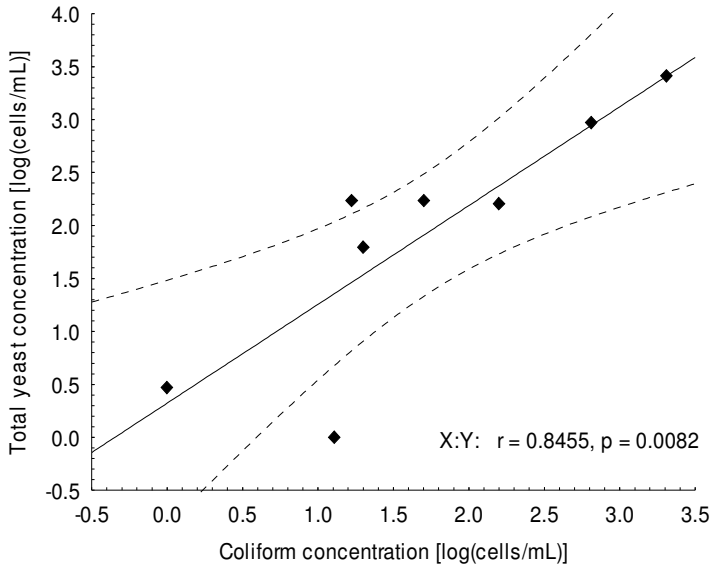

Figure 3a. Correlations between total coliform numbers and total yeast numbers in the clear, flowing (W) zone of the Plankenburg River during the sampling period, spanning the rainy and dry seasons represented in Fig 2. The correlation (r) and p value (p) are both statistically significant.

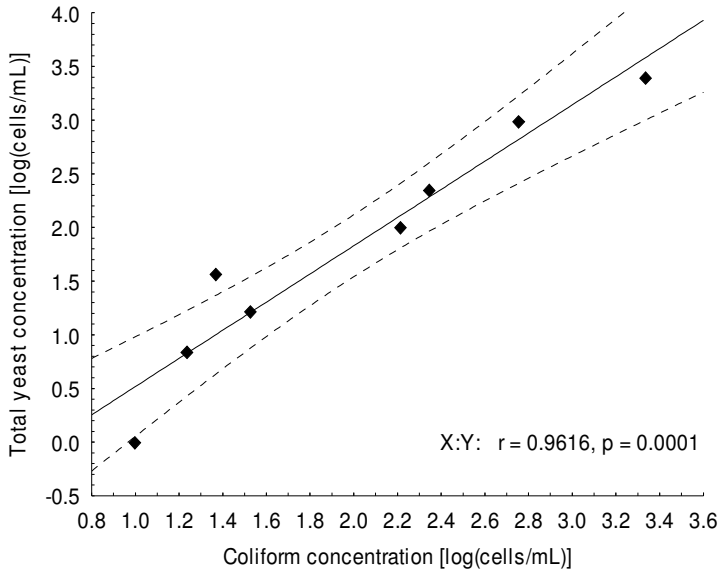

Figure 3b. Correlations between total coliform numbers and total yeast numbers in the rock-filtered (R) zone of the Plankenburg River during the sampling period, spanning the rainy and dry seasons represented in Fig 2. The correlation (r) and p value (p) are both statistically significant.

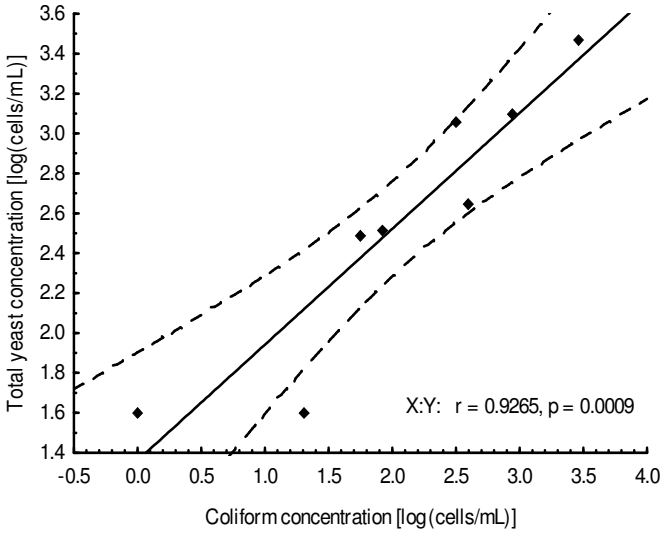

Figure 3c. Correlations between total coliform numbers and total yeast numbers in the plant-filtered (P) zone of the Plankenburg River during the sampling period, spanning the rainy and dry seasons represented in Fig 2. The correlation (r) and p value (p) are both statistically significant.

Table 5. Approximate percentages of *C. albicans* in terms of total yeasts randomly selected from 100 mL sub-samples filtered from the different zones of the Plankenburg River, incubated at 37 ℃ on SGA. *Candida albicans* was semi-quantitatively identified based on Candiselect4 differentiation. The last three rows in bold (D) depict a similar analysis done on the Diep River towards the end of the monitoring period.

Sampling Date	Zone W[1] (%)	Zone R[2] (%)	Zone P[3] (%)
June 2010	0	8	10
July 2010	0	8	9
August 2010	0	3	10
September 2010	0	0	8
October 2010	0	0	10
November 2010	0	0	10
December 2010	0	1	8
January 2011	0	7	9
(D) November 2010	**0**	**0**	**5**
(D) December 2010	**0**	**0**	**5**
(D) January 2011	**0**	**0**	**9**

[1] Zone W: Clear, flowing water *ca*. 50 cm below water surface level.

[2] Zone R: Rock-filtered water *ca*. 50 cm below water surface level.

[3] Zone P: Plant-filtered water *ca*. 50 cm below water surface level.

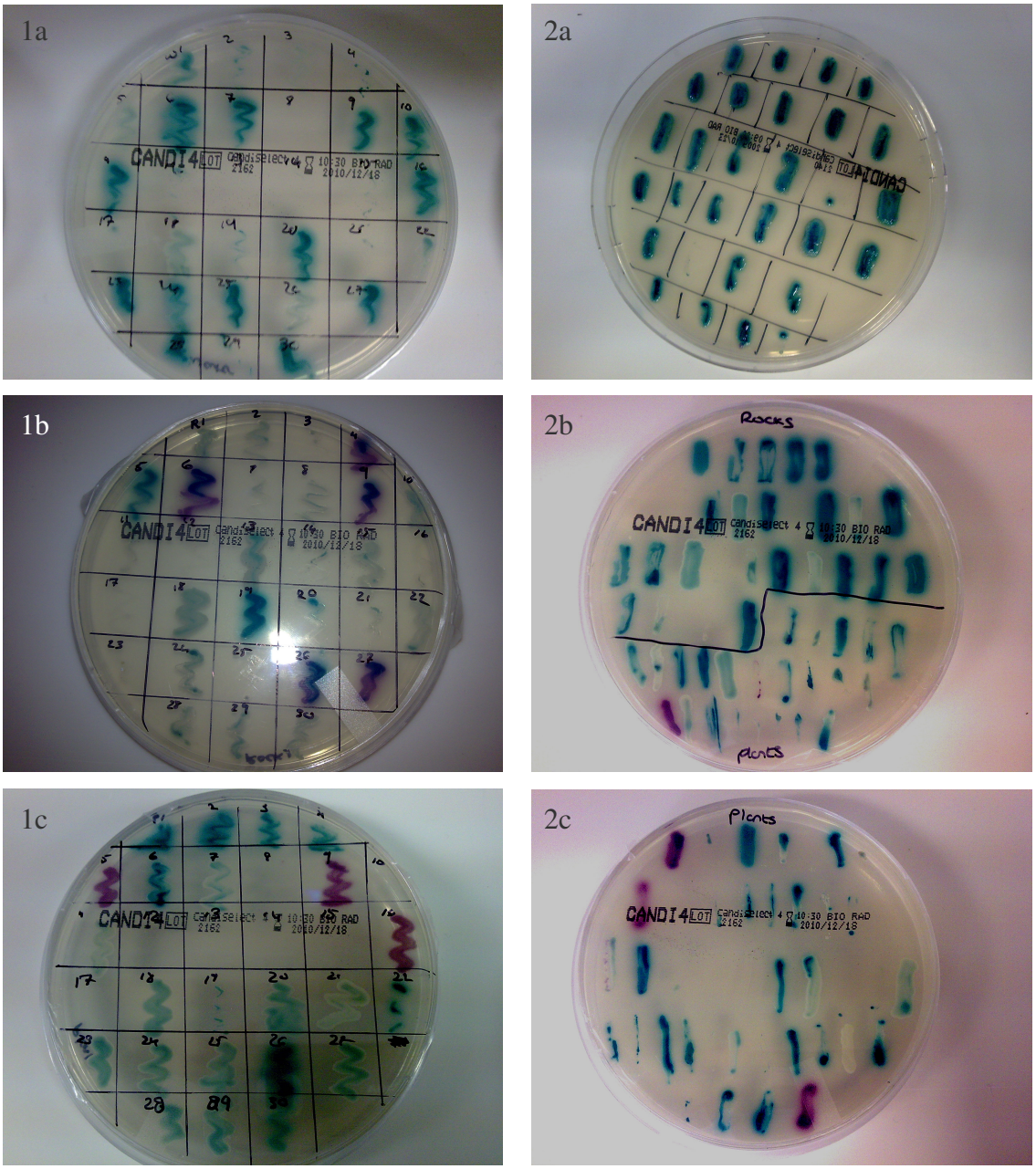

Figure 4. Candiselect4 differentiation of representative yeast species randomly isolated from (a) clear, flowing water (of zone W); (b) rock-filtered water (of zone R) and (c) plant debris-filtered water (of zone P) of the Plankenburg River. 2(b) depicts both rock- and plant-filtered zones, each clearly defined in the image. 1(a-c) depict samples taken in June 2010 (therefore early in the rainy season) whereas 2(a-c) depict samples taken in October 2010 (therefore late in the rainy season). *Candida albicans* species colour purple; *Candida tropicalis*, blue and other yeast species depict no pigmentation.

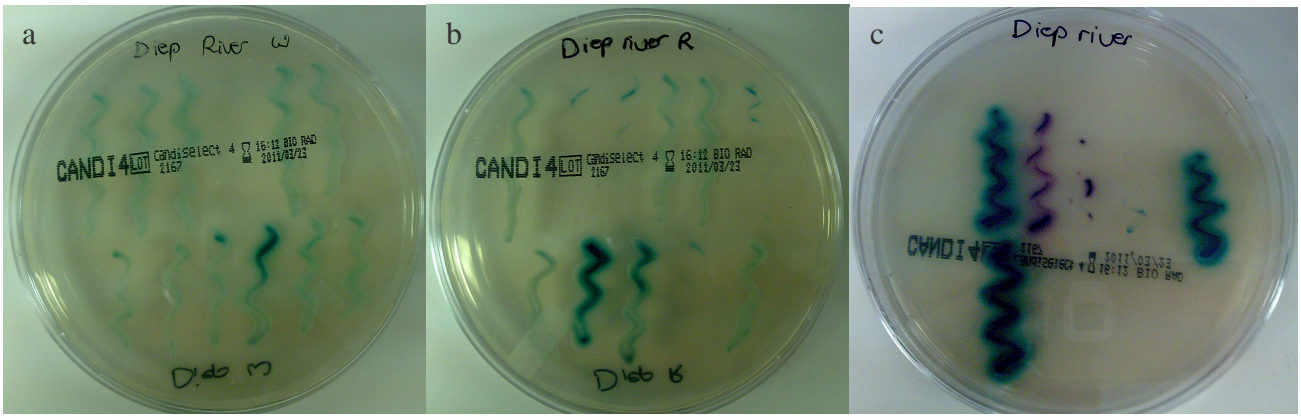

Figure 5. Candiselect4 differentiation of representative yeast species randomly isolated from (a) clear, flowing water (of zone W); (b) rock-filtered water (of zone R) and (c) plant debris-filtered water (of zone P) of the Diep River. Samples were taken in October 2010 (therefore late in the rainy season, corresponding with Figure 4(2a-c)). *Candida albicans* species colour purple; *Candida tropicalis*, blue and other yeast species depict no pigmentation.

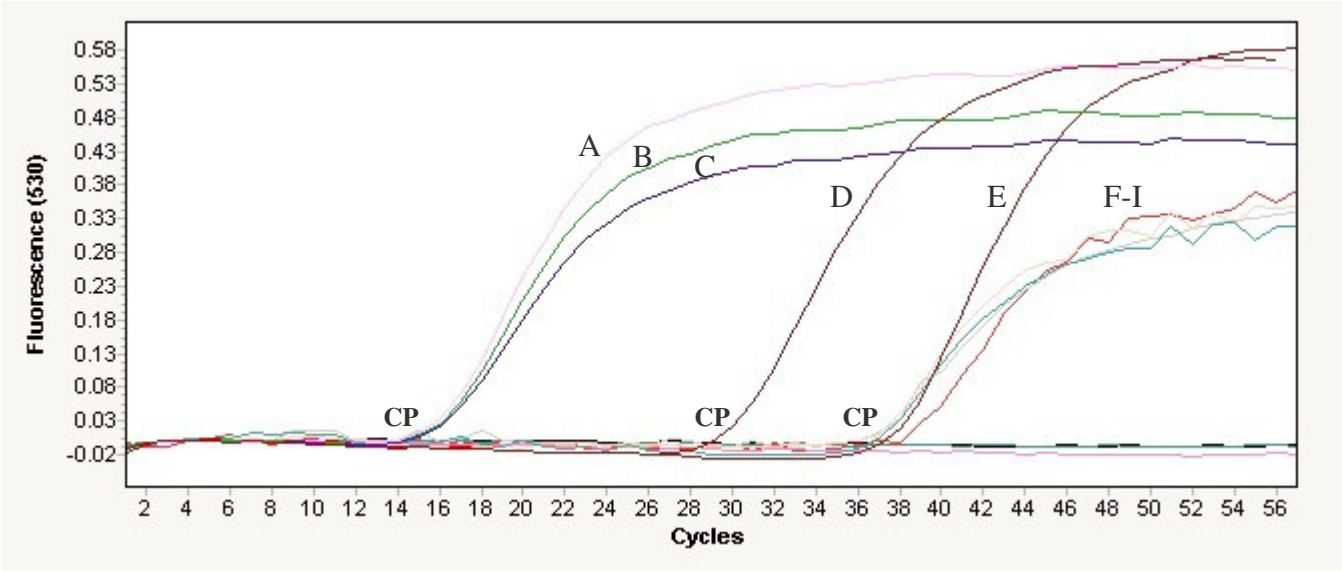

Figure 6. Fluorescence amplification curves of *C. albicans*, detected with qRT-PCR in the three zones (W, R and P) of the Plankenburg River, sampled in November 2010 (therefore late in the rainy season). The P zone is represented by graphs A-C, the W and R zones are represented by graphs F-I (Red and orange – W zone, Green and grey – R zone). Graph D represents the calibrator sample (*C. albicans* CAB 628-1) and graph E represents the reference strain (G. *candidum* CBS 109.12). The crossing points (CP) are used to calculate concentrations in comparison to a standard curve (Appendix D), after normalisation.

Table 6. *Candida albicans* concentrations in the Plankenburg River, analysed by qRT-PCR. The sampling date seasons are indicated in Fig 2(a & b).

Sampling Date	W Zone (cells/mL)	R Zone (cells/mL)	P Zone (cells/mL)
November 2010	0	0	10^3
December 2010	0	0	10^2
January 2011	0	10^2	10^3

2.2.2 *Candida albicans* Identity and Typical Characteristics

From the above discussion, the oxygen-limited, reducing zones in wetlands, which are rich in decaying plant matter, seem to improve the survival of *C. albicans* external to its human host and may provide an ideal external niche for the yeast. In order to assess this risk, a series of *in vitro* studies were carried out on four strains of *C. albicans*. Two were of animal origin (CAB 628-1 and CAB 628-2) and two were clinical (TH 8908 and TH 8912). The morphological and nutrient profile variation of *C. albicans* during general laboratory maintenance demands constant confirmation that any strains dealt with are typical *C. albicans* strains (Rustchenko *et al*., 1994). Chlamydospore formation under semi-anaerobic conditions (Nobile *et al*., 2003), as well as pseudohyphae formation in a protein-rich environment (Buckley and Van Uden, 1963) are characteristic of *C. albicans*. Both phenomena are used in the identification of this yeast, particularly for species differentiation within the genus. These characteristics (Fig 7 & 8), as well as identification on Candiselect4 agar (Fig 9) and, less frequently sequencing (see Appendix A), all regularly confirmed the identity of the strains in question as *C. albicans*. Once, after three months of laboratory maintenance, pseudohyphal formation was not visible in *C. albicans* strain TH 8912 in a protein-rich environment. In addition, CAB 628-2 demonstrated a loss of ability to form chlamydospores, also after months of culture transfers. In each case, the strains were re-inoculated from freeze cultures and experiments were repeated. Otherwise, the strains continuously demonstrated characteristics typical of *C. albicans* and formed the characteristic purple pigment on Candiselect4 plates, before and after each experimental evaluation. Chlamydospore and pseudohyphal formation were also demonstrated in twenty randomly selected *C. albicans* strains isolated from the Plankenburg and Diep Rivers.

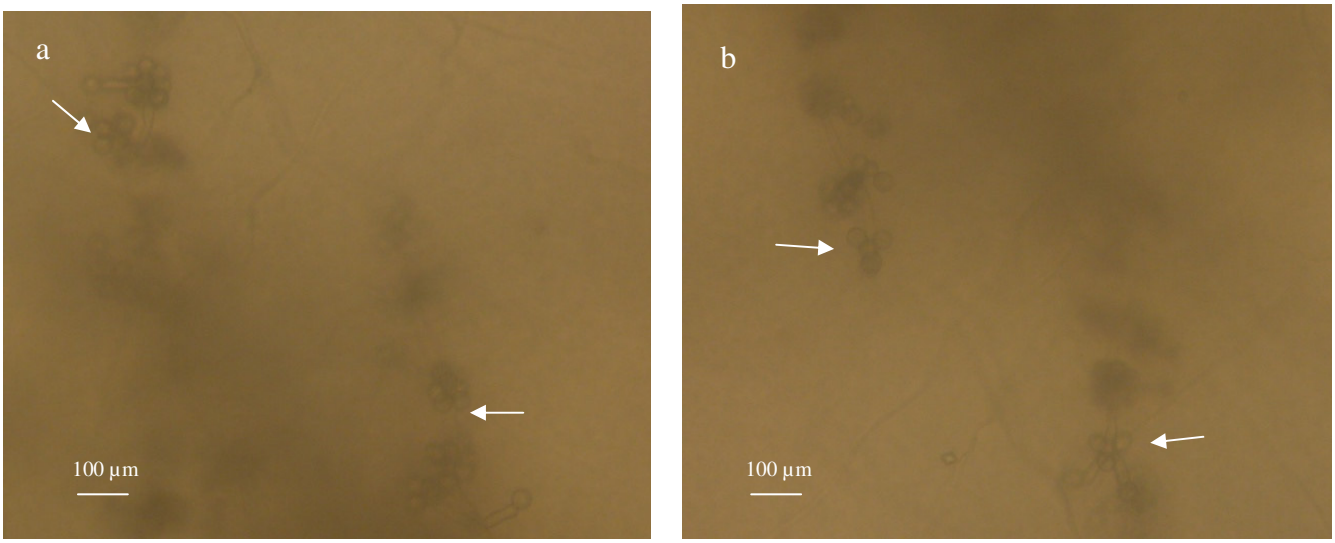

Figure 7. Chlamydospore formation stimulated by the Dalmau inoculation technique in (a) an animal-inhabiting (CAB 628-1) and (b) a clinical (TH 8912) strain of *C. albicans*, tested one year after experimentation began. Arrows indicate individual chlamydospores, much larger than average yeast cells, as demonstrated by comparison with the scale bar.

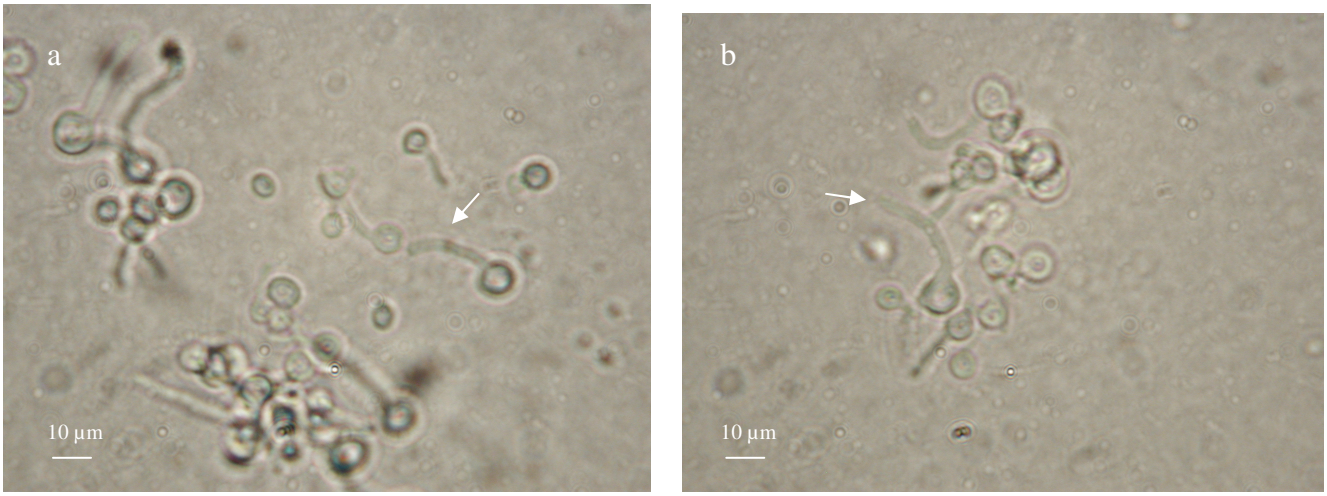

Figure 8. Pseudohyphae formation in a protein-rich egg white environment demonstrated by (a) an animal-inhabiting (CAB 628-2) and (b) a clinical (TH 8908) strain of *C. albicans*, tested 18 months after experimentation began. Arrows indicate individual pseudohyphae.

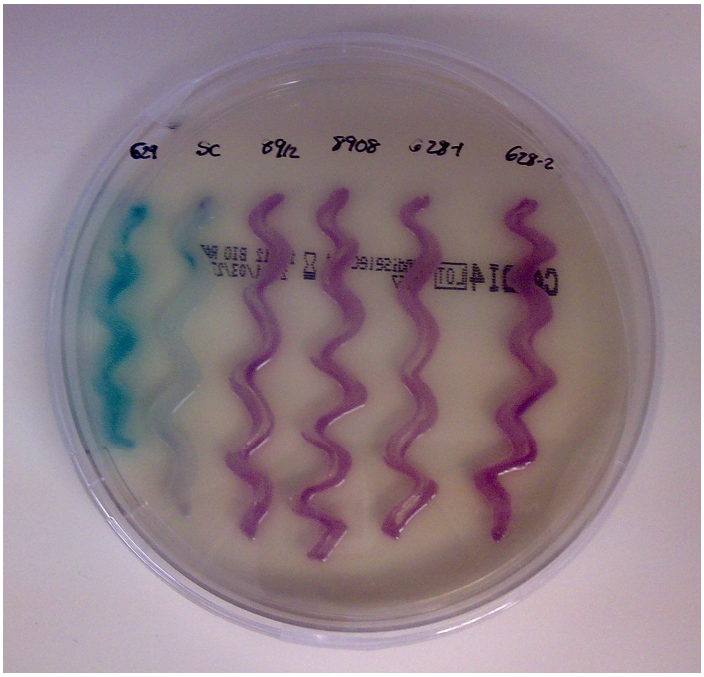

Figure 9. Candiselect4 identification of the four strains of *C. albicans* studied in this work (TH 8912, TH 8908, CAB 628-1 and CAB 628-2) as well as *C. tropicalis* (629) and *S. cerevisiae* (SC). Purple pigmentation is characteristic of *C. albicans* and blue pigmentation is characteristic of *C. tropicalis*, whilst all other yeast species are unpigmented.

2.2.3 *Candida albicans* in the Rhizosphere and in Bulk Soil away from Living Plants

Our discovery of the increased survival capacity of *C. albicans* in the oxygen-limited, plant debris-rich zones of rivers and wetlands initiated an investigation into the means of survival in this region. Oxygen-limitation and low redox potentials were already attributed to the river zone which improved *C. albicans* survival (2.2.1). Therefore, investigations turned to the energy source available to *C. albicans* in this zone. Within wetlands and along river banks, *C. albicans* is exposed to both living and decaying plant matter. Cloete *et al.* (2008) showed that another yeast species, *Cryptococcus laurentii*, is able to grow as a commensal in the rhizosphere of plants. Therefore, it was tested whether *C. albicans* could survive on the carbon and energy sources provided by wetland and crop plant roots. However, neither growth nor survival of *C. albicans* was improved in the rhizosphere, compared to bulk soil away from the root (Fig 10-15). This rhizosphere niche was examined under different soil types, temperatures and plant types. The rhizosphere environment of different crop plants (tomato and maize) had no influence on *C. albicans* growth (Fig 10) in comparison to bulk soil away from the plant. The effect of temperature on the survival and growth of *C. albicans* within the rhizosphere of another

common crop plant (wheat) was evaluated and compared to the bulk soil. At both 24 ℃ and 30 ℃, no significant difference in yeast numbers was observed between the rhizosphere and the bulk soil away from the root (Fig 11). This was visually confirmed on germinating wheat (Fig 12). Different soil types were investigated within the rhizosphere of the wetland macrophytes, *Typha* and *Scirpus*. Composted, loam-like garden soil was compared to wetland soil with a clay-like texture, obtained from the same site as the wetland plants. Wetland mud from this site proved inhibitory to *C. albicans*, with no evident survival of the yeast in the *Typha* and *Scirpus* microcosms after one month (data not shown). On the other hand, *C. albicans* survived in garden soil planted with wetland macrophytes, but again no significant difference was demonstrated between yeast survival in the rhizosphere and survival in the bulk soil away from the root (Fig 13). Microcosm studies attributed the *C. albicans* inhibition of wetland mud to living organisms (Fig 14), since the numbers of the yeast increased notably in sterile mud, while it did not survive two weeks incubation in non-sterile mud microcosms.

It is important to note that although the microbiota of the above-mentioned wetland mud is inhibitory to *C. albicans,* survival of the yeast in this environment was improved in the presence of decaying wetland plant matter (Fig 15). Interestingly, the river bank soil type of the Plankenburg River is similar to loamy garden soil (based on visual evaluation). In contrast, Diep River estuary comprises soil similar to the wetland mud described above (2.1.3.1). The total yeast numbers (data not shown) and *C. albicans* numbers (Table 5) were consistently lower in the Diep River than in the Plankenburg River, possibly due to the inhibitory effect of wetland mud microbiota on the survival of yeast in this environment. Bernhardt *et al*. (1995), Peter and Peter (1988) and Kennedy *et al.* (1987) all emphasised the importance of competition in controlling *C. albicans* proliferation, both in its natural intestinal niche and polluted external environments. This was confirmed in our microcosm studies of wetland mud, which indicated that a major limiting factor for *C. albicans* survival in this environment is the natural microbiota. However, our surveys of polluted rivers show that in the natural environment, *C. albicans* does survive despite such competitive inhibition. This resistance to such inhibition is likely due to the presence of decomposing plant matter as a carbon source and the ability of the yeast to associate with this plant matter, both factors which could increase the competitive ability of the yeast. The decomposing plant matter in the wetland environment may provide energy to improve the competitive ability of the yeast (Saha, 2003; Seybold *et al*., 2002). In addition, *C. albicans* has been shown to be significantly more resistant to environmental stresses within a biofilm formation (Chandra *et al*., 2001a; Chandra *et al*., 2001b; Green *et al*., 2004; Nett *et al*., 2007; Nobile *et al*., 2003), and has been shown to associate with plant matter in an aqueous environment (Elliot and Colwell, 1985)

possibly in a biofilm, which may also improve the survival of the yeast in wetland environments in association with plant matter. This supports the general view on the ecology of this yeast, as literature consistently confirms the adaptation specificity of *C. albicans* to the mammalian host (Fonzi *et al.*, 1993; Hostetter *et al*., 1993; McAlester *et al*., 2008; Rooney and Klein, 2002; Zhang *et al.*, 2000), and exposure to live plants during the evolution of this yeast is thought to have been negligible in comparison to its exposure to decaying plant matter in the vertebrate gastrointestinal tract (Hentges, 1993; Nelson *et al*., 2003).

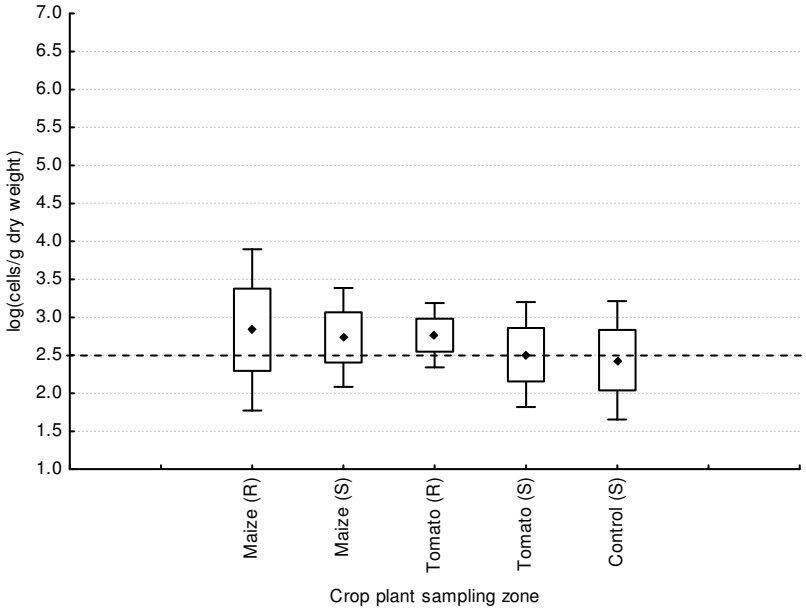

Figure 10. The survival of *C. albicans* CAB 628-1 after two weeks in the rhizosphere zone of common crop flora. Plants were cultivated under glasshouse conditions. (R) refers to the rhizosphere region, (S) to the bulk sand and Control (S) to unplanted sand. *Candida albicans* numbers were determined with six replicates per sample (n=6). Yeast numbers in the respective zones were compared with a Box and Whisker Plot showing mean (♦), mean ± standard deviation (□) and mean ± (1.96 x standard deviation) (⌶). The broken line represents the original log concentration of *C. albicans* in the microcosms (*ca.* 2.6).

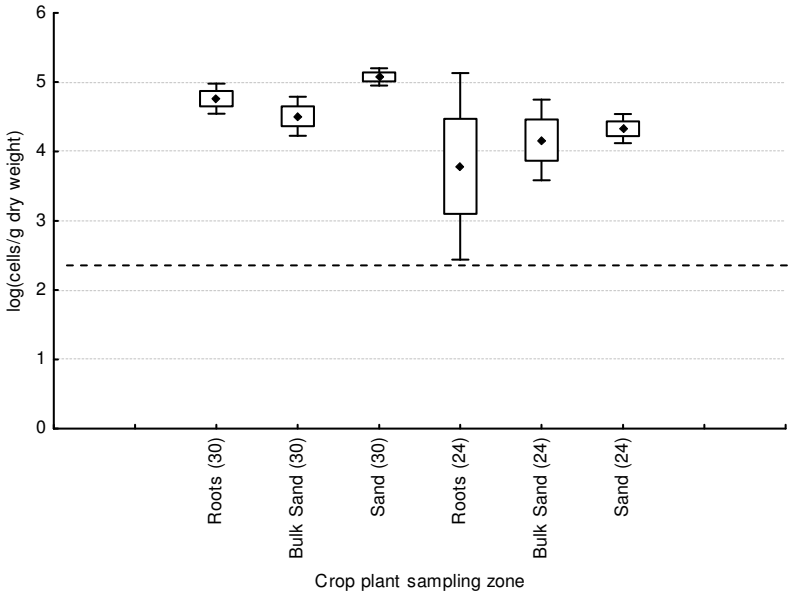

Figure 11. The effect of temperature on the survival of *C. albicans* CAB 628-1 after two weeks in the rhizosphere zone of *Triticum aestivum* (wheat). (Roots) refers to the rhizosphere region, (Bulk Sand) refers to sand away from the root and (Sand) refers to unplanted sand. (30) and (24) refer to the respective temperatures, in ℃. *Candida albicans* numbers were determined with six replicates per sample (n=6). Yeast numbers in the respective zones were compared with a Box and Whisker Plot showing mean (♦), mean ± standard deviation (□) and mean ± (1.96 x standard deviation) (⌶). The broken line represents the original log concentration of *C. albicans* in the microcosms (*ca.* 2.6).

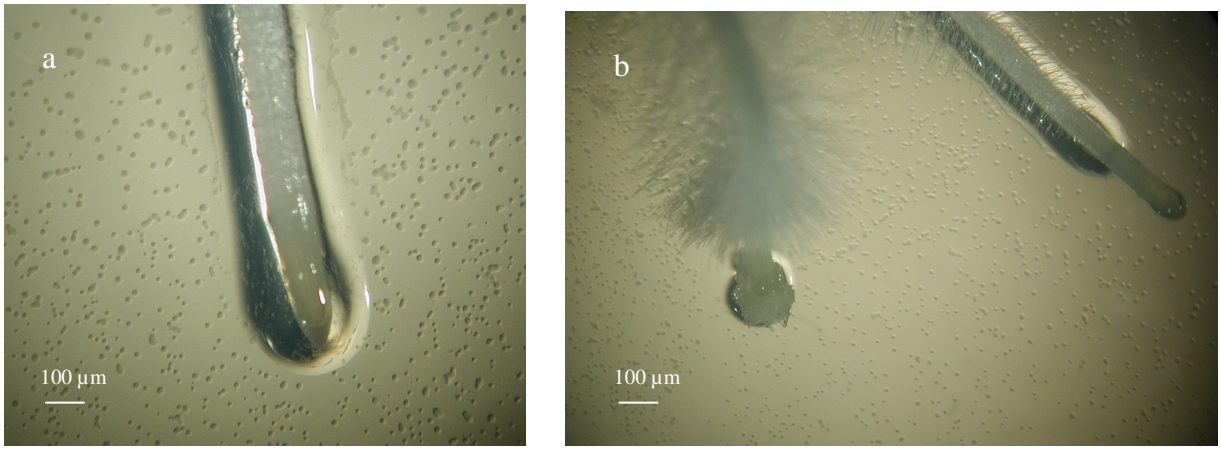

Figure 12. No effect was observed when both (a) an animal-inhabiting (CAB 628-2) and (b) a clinical (TH 8912) strain of *C. albicans* suspended in water agar were exposed to the rhizosphere of surface-sterilised germinating wheat at *ca.* 21 ℃.

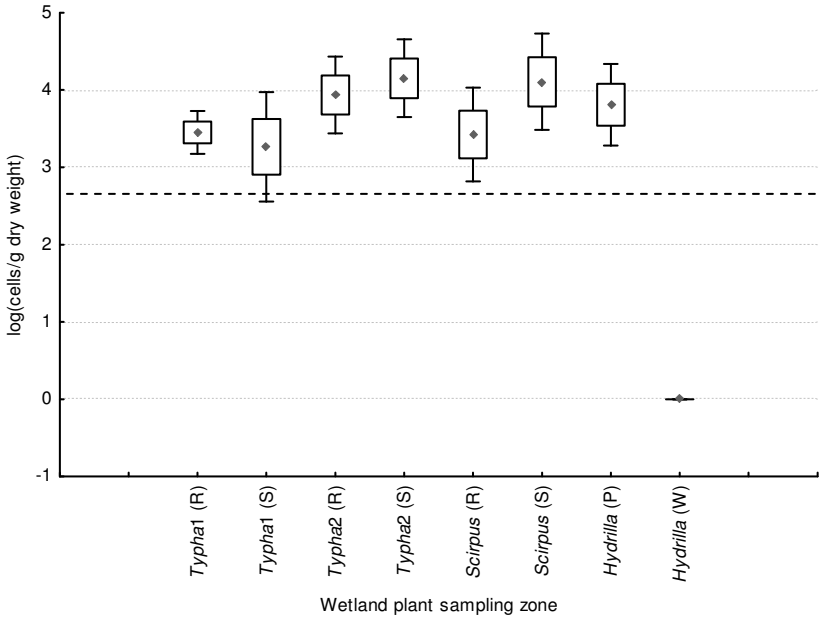

Figure 13. The survival of *C. albicans* CAB 628-1 after one month in the rhizosphere zone of common wetland flora planted in garden soil. Plants were cultivated under glasshouse conditions. (R) refers to the rhizosphere region and (S) refers to the bulk sand away from the roots. With regards to *Hydrilla* (a submerged, drifting plant), (P) refers to the plant and (W) refers to the bulk water away from the plant [log(cells/mL)]. *Candida albicans* numbers were determined with nine replicates per sample (n=9; three replicates of each plant harvested in triplicate). Yeast numbers in the respective zones were compared with a Box and Whisker Plot showing mean (♦), mean ± standard deviation (☐) and mean ± (1.96 x standard deviation) (⌶). The broken line represents the original log concentration of *C. albicans* in the microcosms (*ca.* 2.6).

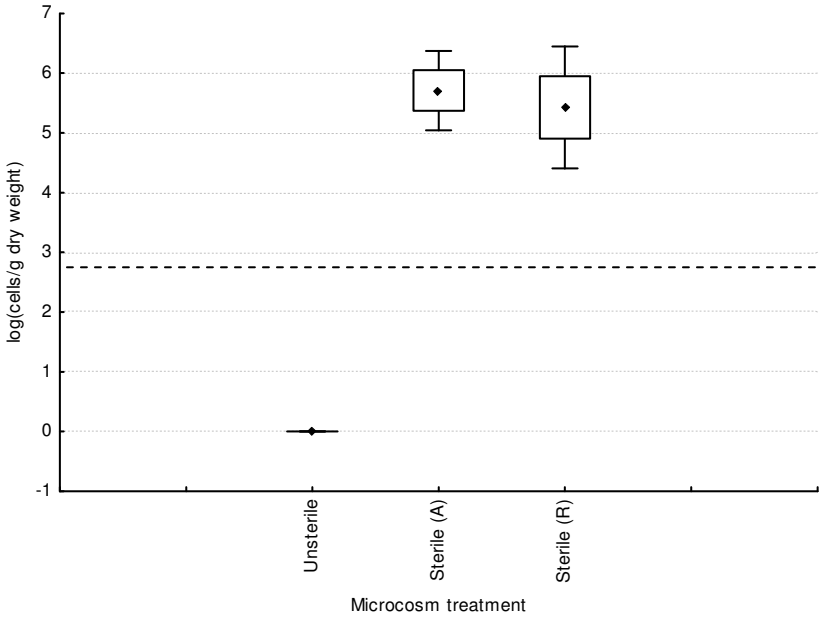

Figure 14. The survival of *C. albicans* TH 8912 after two weeks in wetland mud, comparing sterile and non-sterile conditions. (A) refers to autoclave sterilisation and (R) refers to radiation sterilisation. Three replicates per sample (n=3) were harvested after microcosm incubation at 26 ℃. Yeast numbers were compared with a Box and Whisker Plot showing mean (♦), mean ± standard deviation (□) and mean ± (1.96 x standard deviation) (⌶). The broken line represents the original log concentration of *C. albicans* in the microcosms (*ca.* 2.6).

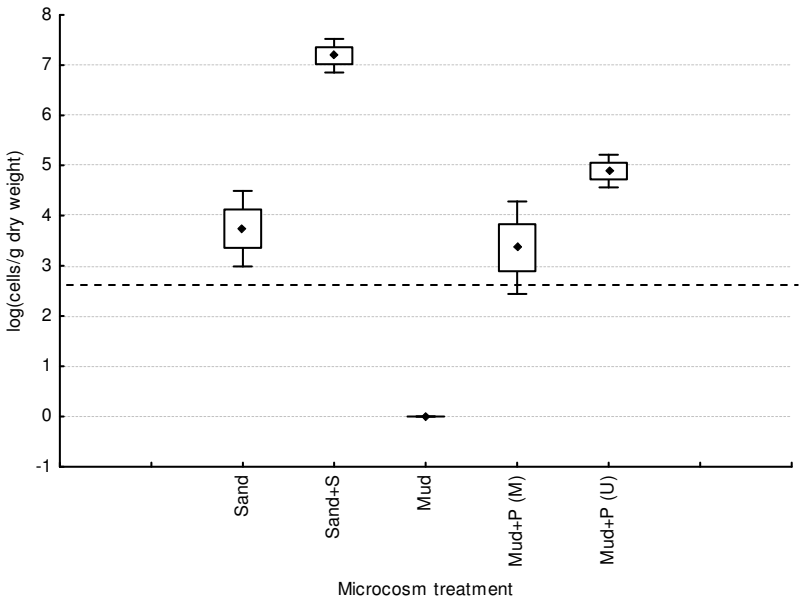

Figure 15. The survival of *C. albicans* CAB 628-1 after two weeks in two different soil types, compared to these soil types supplemented with decaying plant debris (Table 1). (P) refers to wetland plant debris, (S) refers to wheat straw, (M) refers to mixed mud and plant debris and (U) refers to unmixed mud and plant debris (inoculum mixed with plant debris in zone above mud). Three replicates per sample (n=3) were harvested after microcosm incubation at 26 ℃. Yeast numbers were compared with a Box and Whisker Plot showing mean (♦), mean ± standard deviation (□) and mean ± (1.96 x standard deviation) (⌶). The broken line represents the original log concentration of *C. albicans* in the microcosms (*ca.* 2.6).

2.2.4 *Candida albicans* on Decaying Plant Debris

Anaerobic zones in wetlands and along river banks are known to be rich in decaying plant matter, consequently the habitat is reducing and therefore analogous to the gastrointestinal tract from which sewage-borne *C. albicans* most often originates (Hentges, 1993; Nelson *et al*., 2003; Seybold *et al*., 2002). Our survey of natural river banks and wetlands (2.2.1), as well as our *in vitro* microcosm studies on living flora and plant debris (2.2.3), suggest that these oxygen-limited zones may well support growth of *C. albicans*. This concept is strengthened by reports that *C. albicans* is primarily associated with seaweed in polluted oceanic habitats (Elliot and Colwell, 1985). Our work with the submerged wetland macrophyte, *Hydrilla*, also indicated the tendency of the yeast to associate with plant matter in an aquatic environment (Fig 13), possibly affording the yeast biofilm-associated protection. In addition, wetland mud microcosm experiments revealed the increased survival capacity of *C. albicans* in this inhibitory environment in the presence of decaying plant debris, whilst the rhizosphere had no effect on *C. albicans* survival. These results led to a preliminary investigation of the survival of *C. albicans* on decaying plant matter. It was shown that the growth of *C. albicans* on sterile straw and wetland plant matter was consistently improved under aerobic versus anaerobic conditions (Fig 16a & 17a), as expected since aerobic carbohydrate metabolism is more efficient than fermentation (Voet and Voet, 2004). However, under non-sterile conditions growth was consistently improved anaerobically, with significant aerobic growth inhibition, indicating that the yeast competes better under anaerobic conditions (Fig 16b & 17b). The conclusions drawn from these terminally evaluated growth studies were confirmed by monitoring the survival and growth of *C. albicans* CAB 628-1 in a month-long microcosm study (Fig 18-21). Within the microcosms, in sterile conditions yeast numbers increased exponentially during the first six days and reached an aerobic and anaerobic stationary phase, remaining at *ca.* log 6 and log 5 cells/g dry weight of plant matter respectively (Fig 18 & 19). Under non-sterile conditions *C. albicans* CAB 628-1 was completely inhibited aerobically, with the cell count dropping to zero by day 24 (Fig 20). In contrast and most pertinently, although yeast numbers dropped during the initial incubation period under non-sterile anaerobic conditions (Fig 21); the population recovered by day 36, demonstrating an increased competitive capacity on plant debris under anaerobic conditions. The *C. albicans* population could survive and growth was even demonstrated by day 42 under these conditions (Fig 21). Therefore, the contention is supported that *C. albicans* competes well on decaying plant matter under oxygen-limited conditions similar to those of the gastrointestinal tract. The largely saprophytic nature of fungi (Kerridge, 1993) explains this tendency; rather than that of a commensal, mycorrhizal relationship with living plant matter. In addition, the suggestion of De Hoog

(2000) is supported: that an external habitat could be established by locating the environment where both the reproductive structures and assimilative thallus are formed, as well as being attributed to a few pertinent characteristics analogous to those of the host niche.

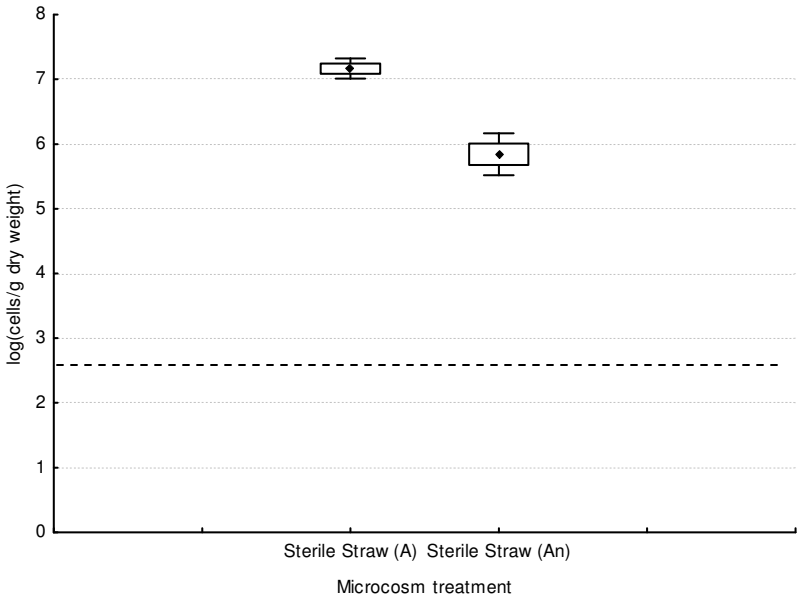

Figure 16a. Growth of *C. albicans* CAB 628-1 after two weeks on sterile wheat straw under aerobic (A) and anaerobic (An) conditions. The results are the means (♦) of three repetitions of each treatment in microcosms incubated at 26 °C. Mean ± standard deviation (□) and mean ± (1.96 x standard deviation) (⌶) are depicted. The broken line represents the original log concentration of *C. albicans* in the microcosms (*ca.* 2.6).

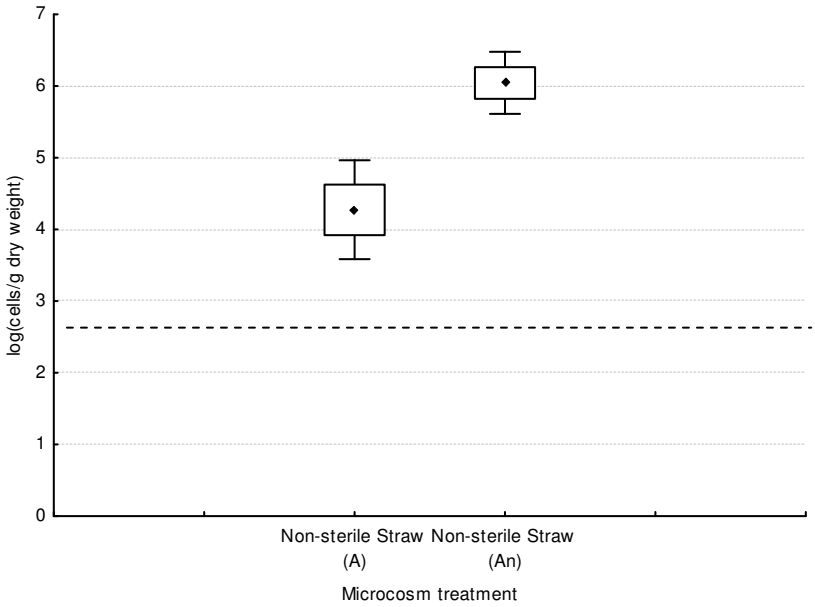

Figure 16b. Growth of *C. albicans* CAB 628-1 after two weeks on non-sterile wheat straw under aerobic (A) and anaerobic (An) conditions. The results are the means (♦) of three repetitions of each treatment in microcosms incubated at 26 °C. Mean ± standard deviation (□) and mean ± (1.96 x standard deviation) (⌶) are depicted. The broken line represents the original log concentration of *C. albicans* in the microcosms (*ca.* 2.6).

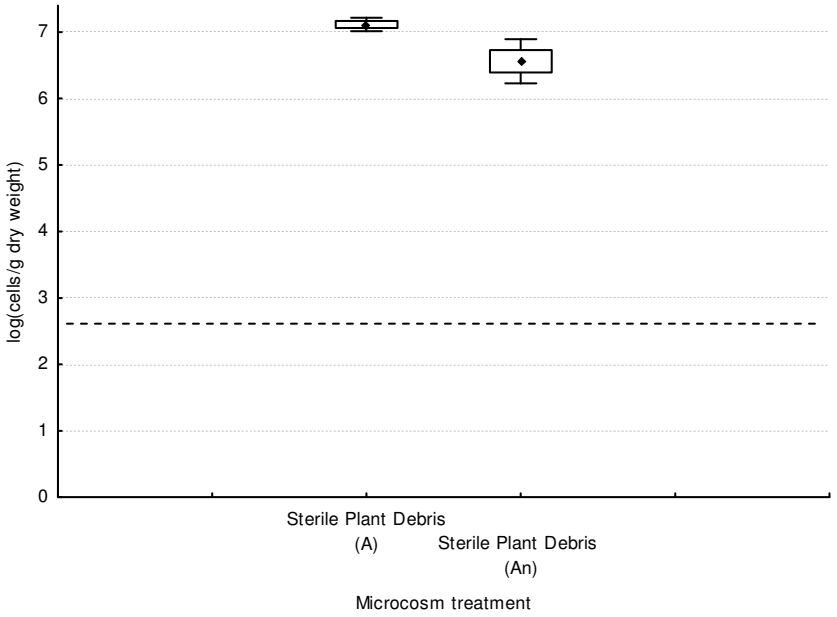

Figure 17a. Growth of *C. albicans* CAB 628-1 after two weeks on sterile wetland plant matter under aerobic (A) and anaerobic (An) conditions. The results are the means (♦) of three repetitions of each treatment in microcosms incubated at 26 ℃. Mean ± standard deviation (□) and mean ± (1.96 x standard deviation) (⌶) are depicted. The broken line represents the original log concentration of *C. albicans* in the microcosms (*ca.* 2.6).

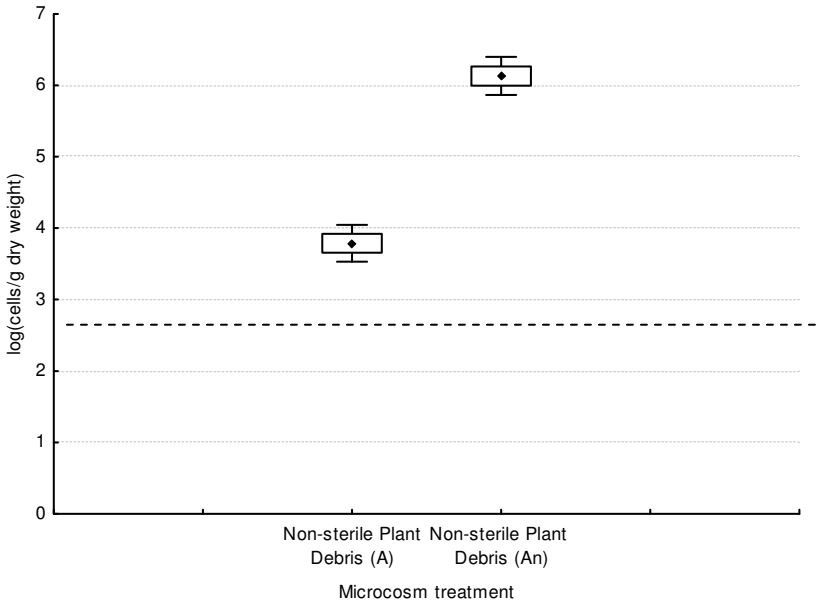

Figure 17b. Growth of *C. albicans* CAB 628-1 after two weeks on non-sterile wetland plant matter under aerobic (A) and anaerobic (An) conditions. The results are the means (♦) of three repetitions of each treatment in microcosms incubated at 26 ℃. Mean ± standard deviation (□) and mean ± (1.96 x standard deviation) (⌶) are depicted. The broken line represents the original log concentration of *C. albicans* in the microcosms (*ca.* 2.6).

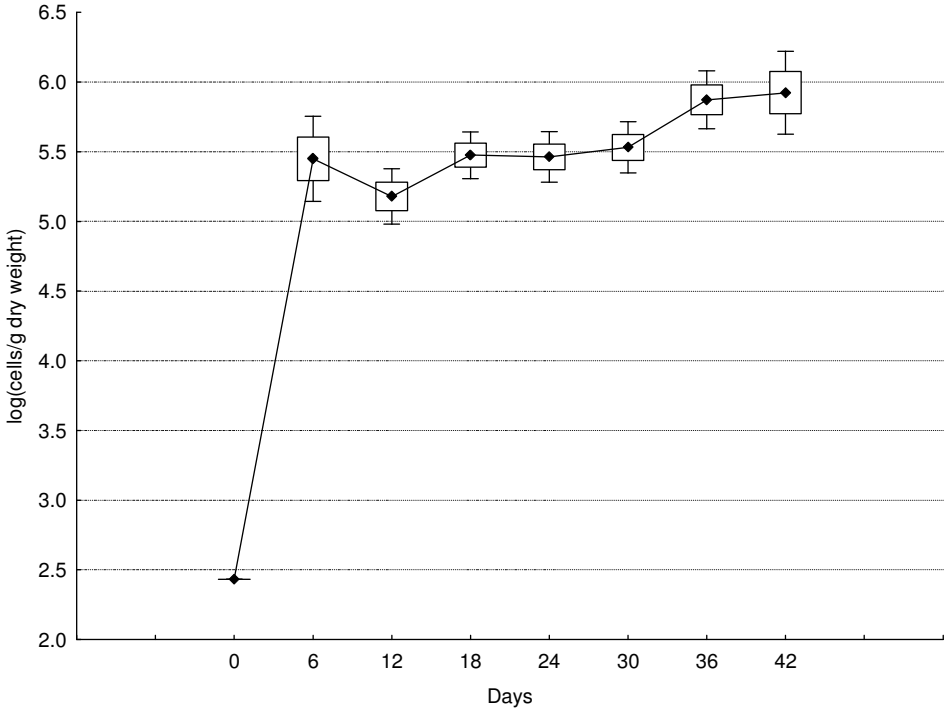

Figure 18. Growth of *C. albicans* CAB 628-1 on sterile wetland plant matter under aerobic conditions over a period of 42 days. The results are the means (♦) of eight repetitions in microcosms incubated at 26 ℃. Mean ± standard deviation (□) and mean ± (1.96 x standard deviation) (⌶) are depicted.

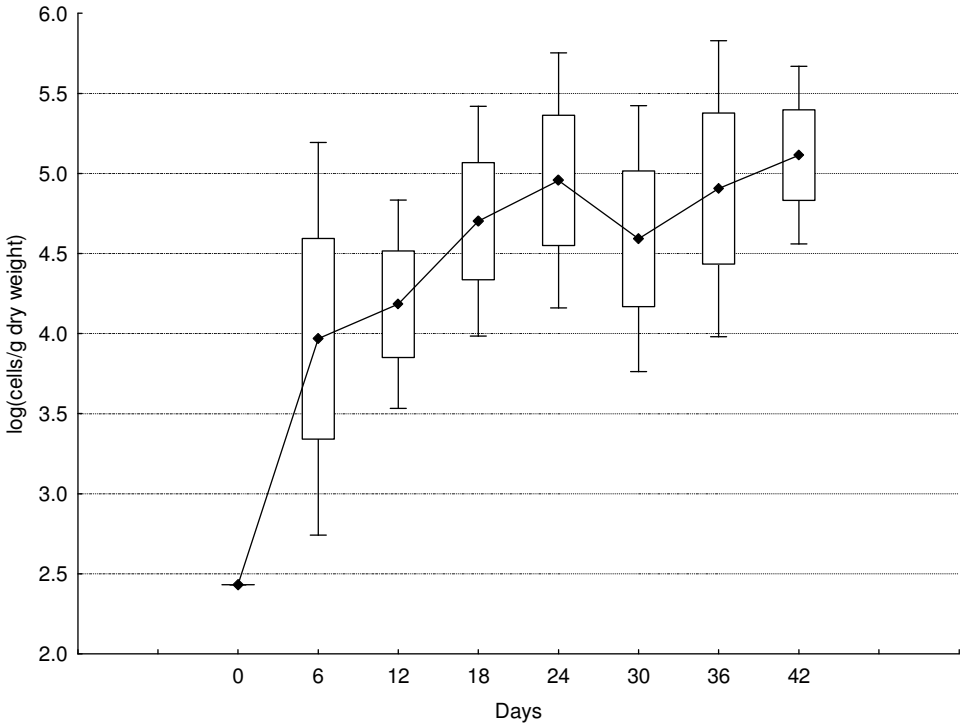

Figure 19. Growth of *C. albicans* CAB 628-1 on sterile wetland plant matter under anaerobic conditions over a period of 42 days. The results are the means (♦) of eight repetitions in microcosms incubated at 26 ℃. Mean ± standard deviation (□) and mean ± (1.96 x standard deviation) (⌶) are depicted.

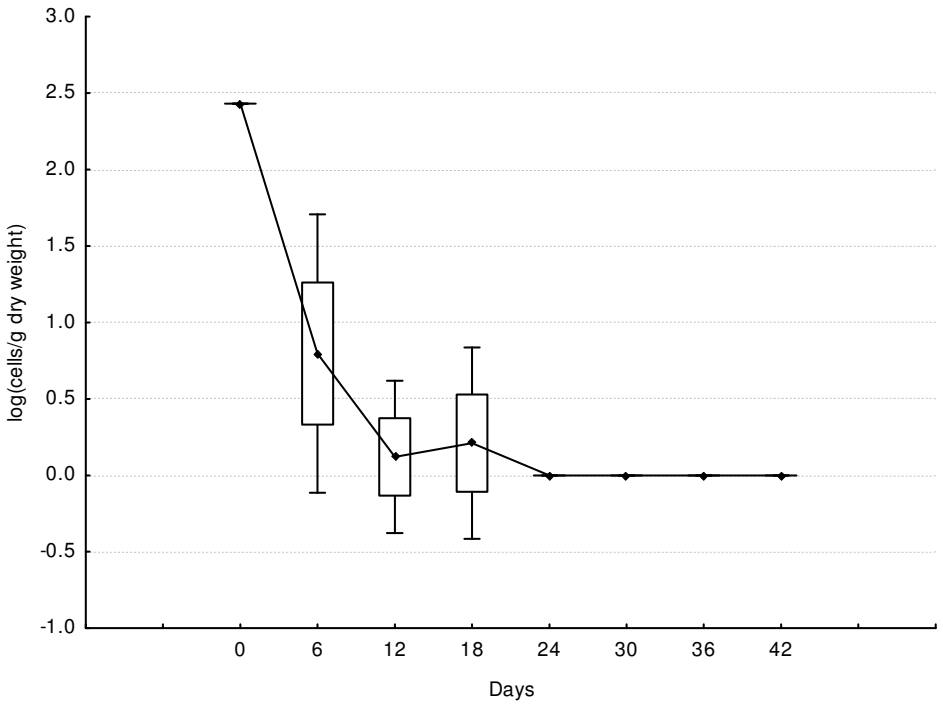

Figure 20. Survival of *C. albicans* CAB 628-1 on non-sterile wetland plant matter under aerobic conditions over a period of 42 days. The results are the means (♦) of eight repetitions in microcosms incubated at 26 ℃. Mean ± standard deviation (□) and mean ± (1.96 x standard deviation) (⌶) are depicted.

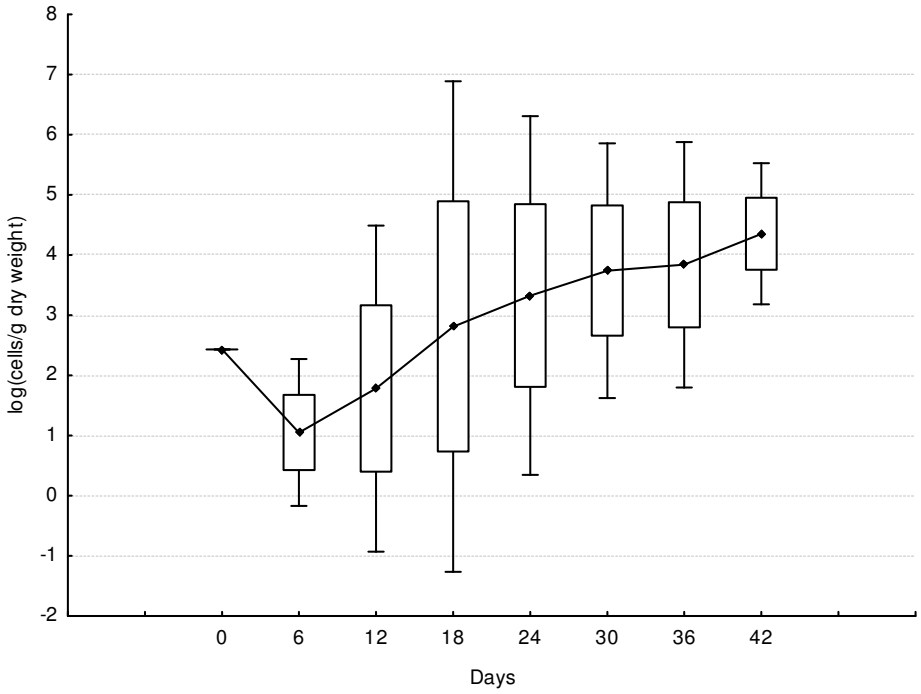

Figure 21. Growth of *C. albicans* CAB 628-1 on non-sterile wetland plant matter under anaerobic conditions over a period of 42 days. The results are the means (♦) of eight repetitions in microcosms incubated at 26 ℃. Mean ± standard deviation (□) and mean ± (1.96 x standard deviation) (⌶) are depicted.

2.2.5 Elucidation of the Growth Substrates of *C. albicans* in Wetland Plant Debris Extract

Plant matter degradation provides the major carbon and energy source in the reducing, oxygen-limited environment to which *C. albicans* is exposed in wetlands and along river banks. Decaying plant matter provides lignocellulose, cellulose and simple mono- and disaccharides as potentially available energy and carbon sources. Simple carbohydrates are the most likely source of energy for *C. albicans* (Saha, 2003; Van Uden, 1960) and it has been repeatedly reported that the yeast is unable to assimilate the simplest cellulosic product, cellobiose (Chandra *et al*., 2001a; Hunter and Fraser, 1989; Kurtzman and Fell, 2000; Rustchenko *et al*., 1997). However, the utilisation of cellulose would afford *C. albicans* a competitive advantage within the wetland environment and this yeast has demonstrated the ability to adapt its nutrient utilisation profile (Rustchenko *et al.*, 1997). Therefore, the utilisation of all the carbon sources comprising decaying wetland plant matter was investigated.

2.2.5.1 Complex carbohydrates

Candida albicans has been shown to ferment glucose, producing acid as a by-product, particularly within the oral environment (Nikawa *et al*., 1994; Samaranayake *et al*., 2006). We investigated the possibility that this proton-rich environment may assist in the degradation of cellulosic substrates, making simple carbohydrates available to the yeast. We confirmed this acid production (Fig 22); however, acid production was slower and less effective anaerobically and was not demonstrated to be connected to any cellulose utilisation. Cellobiose (data not shown) and CMC utilisation (Fig 23) was absent in all four *C. albicans* strains (CAB 628-1, CAB 628-2, TH 8908, TH 8912), as well as in strains removed from the Plankenburg and Diep River environments. This confirmed the work of the authors mentioned above (Section 2.2.5). CMC utilisation was also evaluated in the presence of low concentrations of mono- and disaccharides, as this is similar to the oral environment and was previously demonstrated to affect carbon utilisation profiles (Nikawa *et al*., 1994; Pearson *et al*., 1990; Samaranayake *et al*., 2006). However, this had no influence on the utilisation of cellulose (Fig 23). These results indicate that the drop in pH caused by *C. albicans* does not render it able to utilise cellulose. Similarly, an ANKOM neutral detergent fibre analysis demonstrated the incapacity of *C. albicans* for anaerobic lignocellulosic fibre degradation (Fig 24), with a similar trend under aerobic conditions. Thus, the results indicated that complex carbohydrates are unavailable to the *C. albicans* strains evaluated in this study; therefore, simple mono- and disaccharides released as natural

degradation products from lignocellulose seem to be the major potential carbon source in a wetland environment.

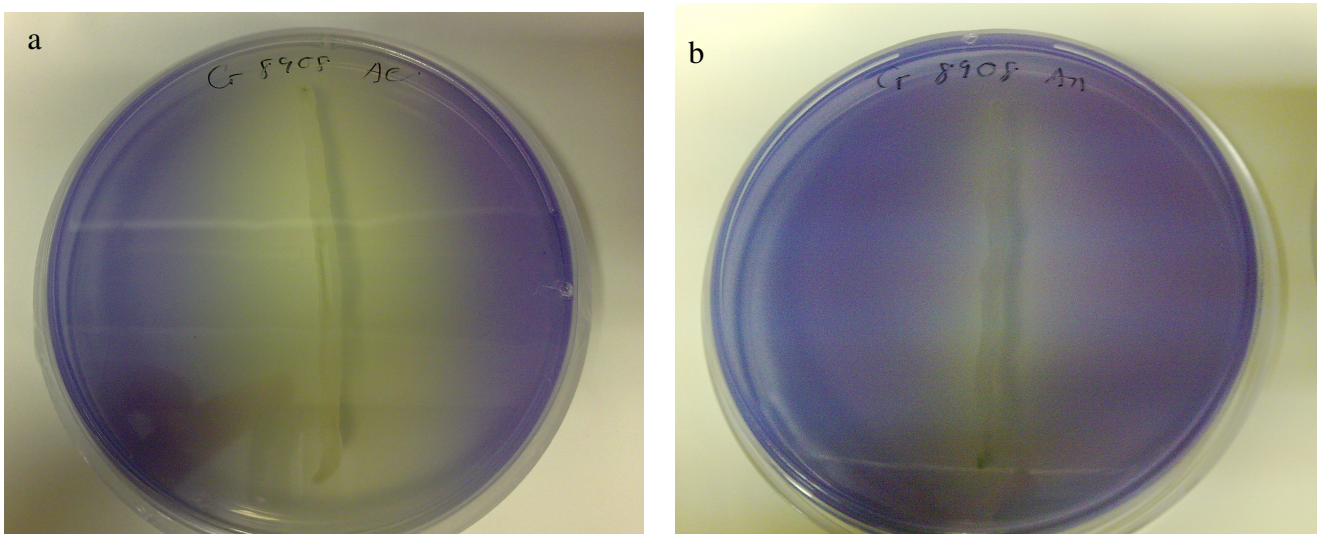

Figure 22. Yellow pigmentation depicts (a) aerobic (at 24 h) and (b) anaerobic (at 72 h) acid production of a clinical strain of *C. albicans* (TH 8908) on YNB agar, supplemented with glucose and bromophenol blue.

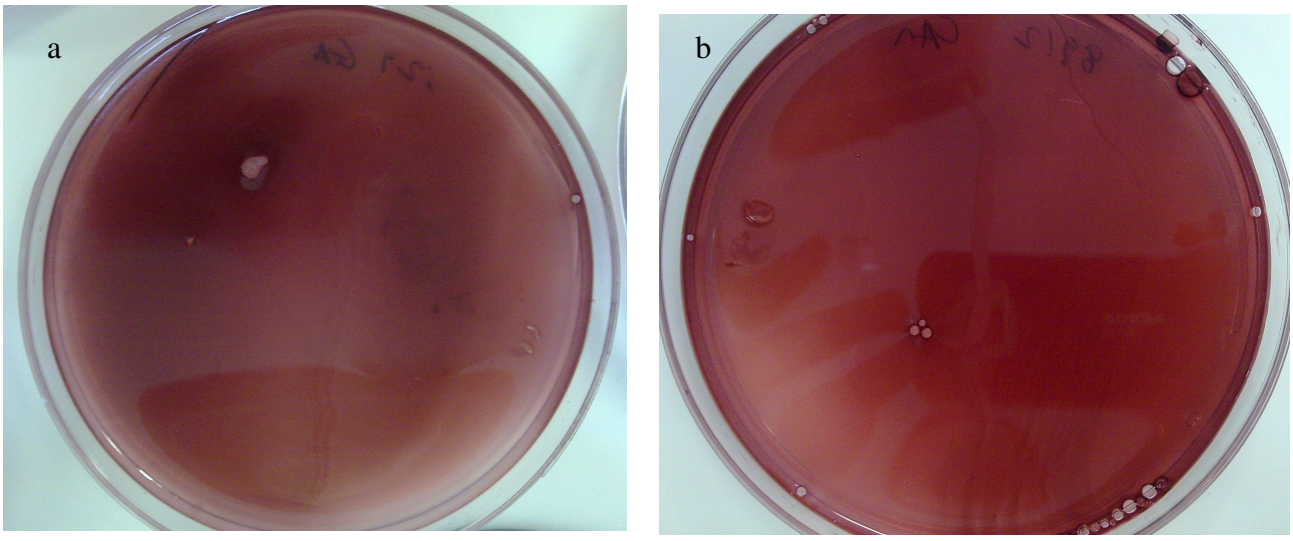

Figure 23. No clear zones are evident on the plates depicted above. Therefore the absence of CMC utilisation (in the presence of glucose) by (a) an animal-inhabiting (CAB 628-1) and (b) a clinical (TH 8912) strain of *C. albicans* is clear under anaerobic conditions.

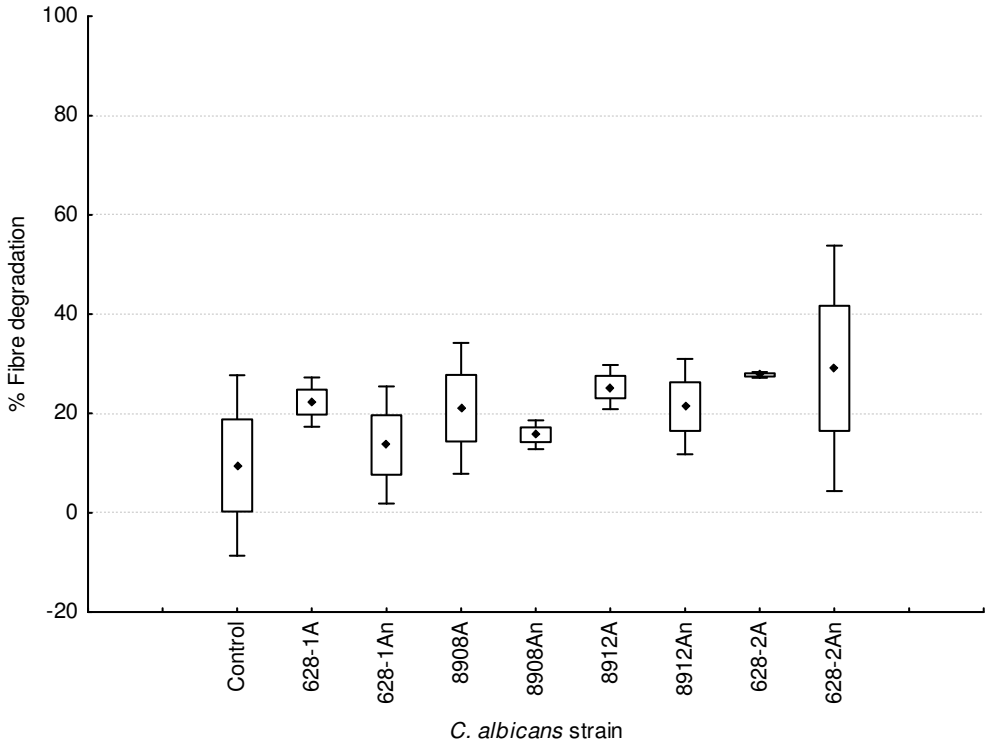

Figure 24. Aerobic (A) and anaerobic (An) fibre degradation capacity of the four strains of *C. albicans* (CAB 628-1, CAB 628-2, TH 8908, TH 8912) in comparison to an uninoculated control. The results are the means (♦) of three repetitions of microcosms incubated at 30 ℃. Mean ± standard deviation (□) and mean ± (1.96 x standard deviation) (⌶) are depicted.

2.2.5.2 Mono- and disaccharide assimilation

The ability of *C. albicans* to aerobically metabolise a range of mono- and disaccharides; as well as ferment glucose, galactose and sucrose; has been reported (Kurtzman and Fell, 2000). In addition, Rustchenko's research has established that this nutrient profile is flexible (Rustchenko *et al.*, 1994; Rustchenko *et al.*, 1997). Therefore, we employed HPLC to tentatively determine the presence of such mono- and disaccharides in wetland plant debris extract, and evaluate the ability of *C. albicans* (CAB 628-1) to anaerobically assimilate these carbohydrates. The extract constituted significant (30-90 ppm) concentrations of glucose, fructose and arabinose (Fig 25); whereas xylose and sucrose were evident at much lower levels (<30 ppm). However, only glucose and fructose appeared to be utilised by CAB 628-1 under anaerobic conditions during the incubation period. GC-MS confirmed this wetland plant debris carbohydrate depletion profile in *C. albicans* strains CAB 628-1, CAB 628-2, TH 8908 and TH 8912 (Fig 26a-e). Interestingly, very little variation was observed among the profiles

of the strains that were tested, although they originated from different sources (CAB 628-1 and CAB 628-2 from animals; TH 8908 and TH 8912 from clinical samples). In this plant debris extract, similar to that analysed with HPLC, glucose and galactose were predominant (> 10 ppm), although all concentrations appeared lower than in the analogous extract analysed with HPLC (Fig 25 & 26a-e). Nevertheless, all strains again demonstrated the capacity to anaerobically assimilate glucose and fructose present in the wetland plant debris extract (Fig 26a & b), whereas all other sugars seemed to remain in the extract after incubation with *C. albicans* (Fig 26c-e). These results were confirmed since our *C. albicans* strains were all shown to grow anaerobically on glucose and fructose as sole carbon source; while arabinose, galactose, sucrose and xylose were not assimilated under these conditions (2.1.5.5 Anaerobic growth on single mono- and disaccharides). In addition, a commercial analysis of the nitrogenous compounds in the samples analysed with HPLC also revealed an approximate decrease in total nitrogen content from 0.16 % (m/m) to 0.006 % (m/m), indicating nitrogen utilisation during anaerobic growth of *C. albicans* on the plant extract (Table 7). Particularly, NH_4^+ and NO_3^- seemed to be assimilated. Therefore, we demonstrated potential nutrient sources for *C. albicans* within the oxygen-limited, reducing environment of wetland and river bank microhabitats.

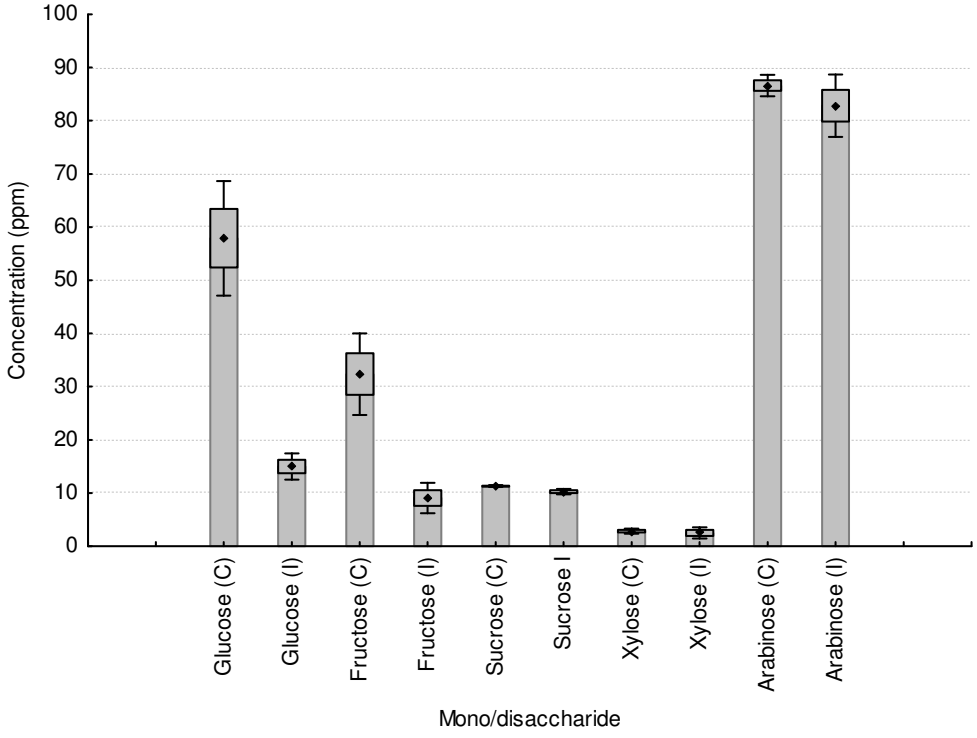

Figure 25. Concentrations of a range of mono- and disaccharides, determined using HPLC, remaining in a wetland plant debris extract after anaerobic culturing with *C. albicans* CAB 628-1 (I), in comparison with an uninoculated control (C). The yeast was cultured in the plant extract for *ca.* 7 h at 30 °C. The results are the means (♦) of three repetitions. Mean ± standard deviation (□) and mean ± (1.96 x standard deviation) (⌶) are depicted.

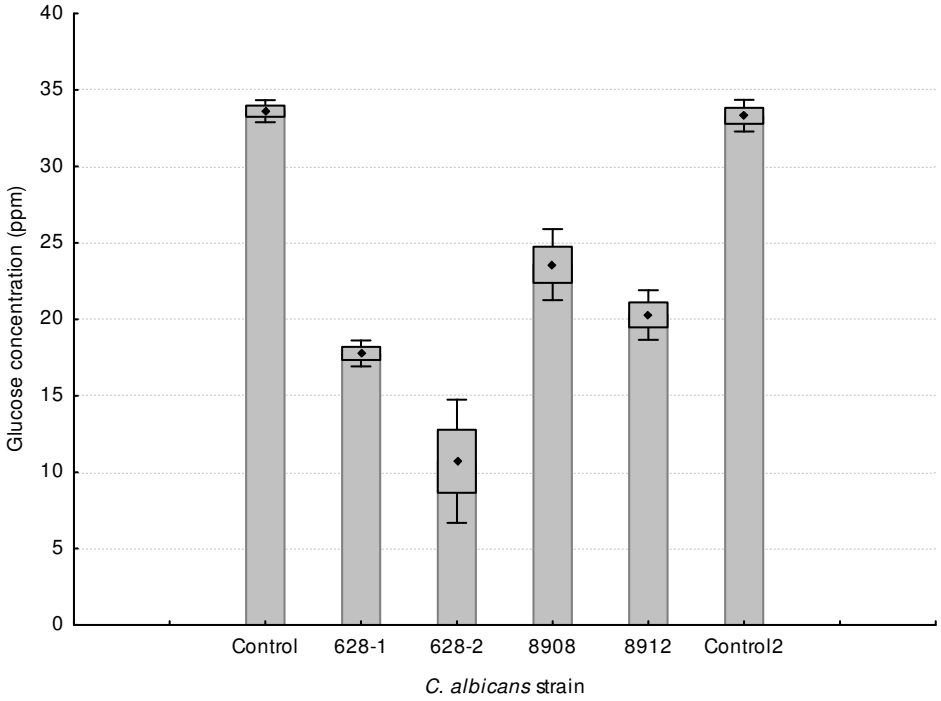

Figure 26a. Glucose concentrations, determined using GC-MS, remaining in a wetland plant debris extract after anaerobic culturing with *C. albicans* strains (CAB 628-1, CAB 628-2; TH 8908; TH 8912), in comparison with uninoculated controls. The yeast was cultured in the plant extract for *ca*. 7 h at 30 ℃. The results are the means (♦) of three repetitions. Mean ± standard deviation (□) and mean ± (1.96 x standard deviation) (⌶) are depicted.

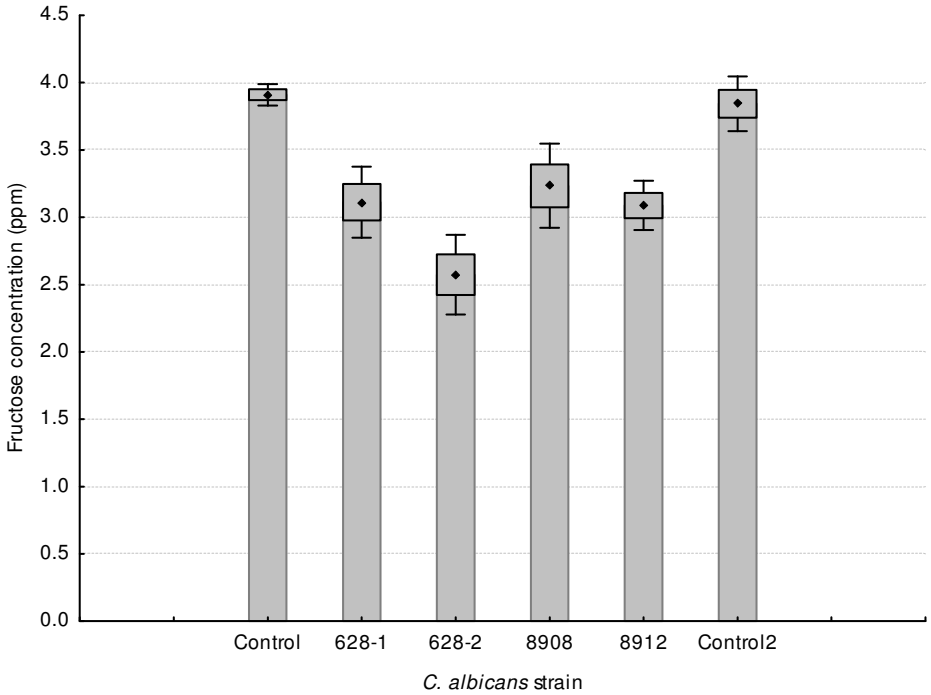

Figure 26b. Fructose concentrations, determined using GC-MS, remaining in a wetland plant debris extract after anaerobic culturing with *C. albicans* strains (CAB 628-1, CAB 628-2; TH 8908; TH 8912), in comparison with uninoculated controls. The yeast was cultured in the plant extract for *ca*. 7 h at 30 ℃. The results are the means (♦) of three repetitions. Mean ± standard deviation (□) and mean ± (1.96 x standard deviation) (⌶) are depicted.

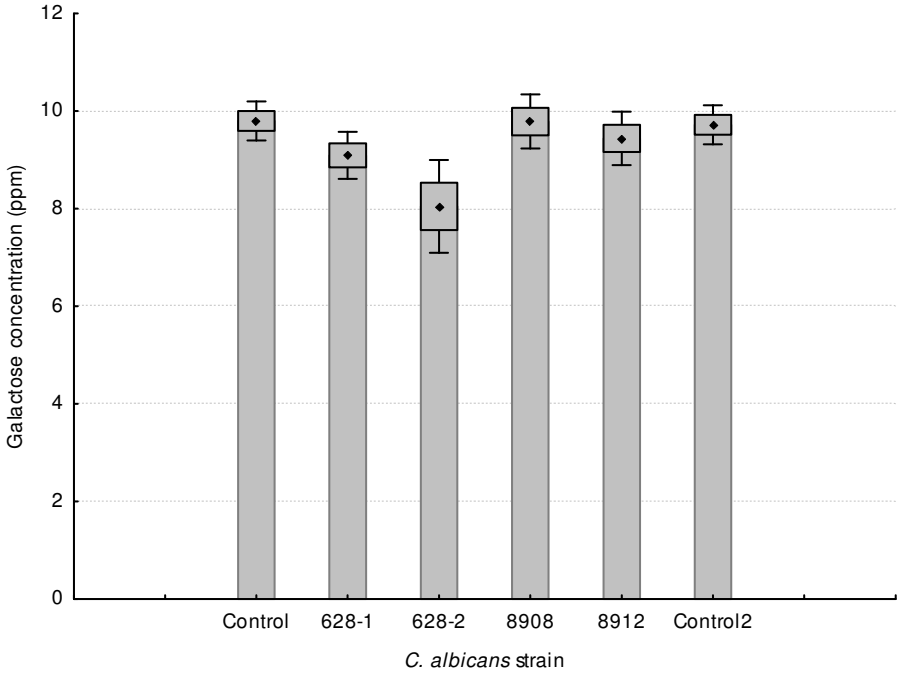

Figure 26c. Galactose concentrations, determined using GC-MS, remaining in a wetland plant debris extract after anaerobic culturing with *C. albicans* strains (CAB 628-1, CAB 628-2; TH 8908; TH 8912), in comparison with uninoculated controls. The yeast was cultured in the plant extract for *ca*. 7 h at 30 °C. The results are the means (♦) of three repetitions. Mean ± standard deviation (□) and mean ± (1.96 x standard deviation) (⌶) are depicted.

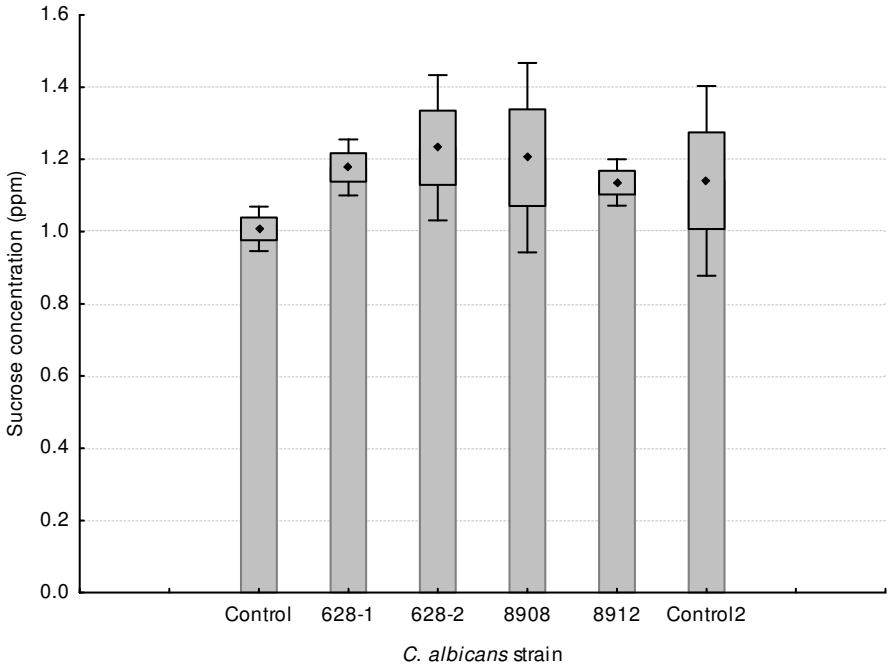

Figure 26d. Sucrose concentrations, determined using GC-MS, remaining in a wetland plant debris extract after anaerobic culturing with *C. albicans* strains (CAB 628-1, CAB 628-2; TH 8908; TH 8912), in comparison with uninoculated controls. The yeast was cultured in the plant extract for *ca.* 7 h at 30 °C. The results are the means (♦) of three repetitions. Mean ± standard deviation (□) and mean ± (1.96 x standard deviation) (⌶) are depicted.

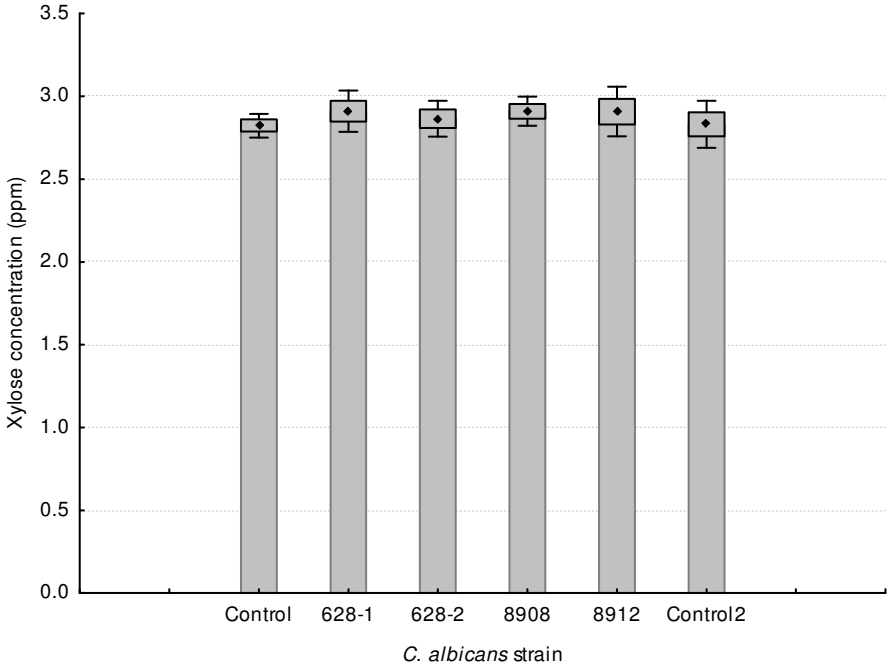

Figure 26e. Xylose concentrations, determined using GC-MS, remaining in a wetland plant debris extract after anaerobic culturing with *C. albicans* strains (CAB 628-1, CAB 628-2; TH 8908; TH 8912), in comparison with uninoculated controls. The yeast was cultured in the plant extract for *ca.* 7 h at 30 ℃. The results are the means (♦) of three repetitions. Mean ± standard deviation (□) and mean ± (1.96 x standard deviation) (⌶) are depicted.

Table 7. A commercial analysis evaluating the assimilation of nitrogenous compounds in the samples analysed with HPLC (Fig 25). Control represents an uninoculated wetland plant debris extract, incubated anaerobically for *ca.* 7 h at 30 ℃. Inoculated represents wetland plant debris extract incubated anaerobically with *C. albicans* CAB 628-1 for *ca.* 7 h at 30 ℃.

Sample	N (%)	NH_4-N (mg/kg)	NO_3-N (mg/kg)	NO_2-N (mg/kg)
Control	0.16	17.46	1.30	0.75
Inoculated	0.06	16.42	1.04	0.74

2.3 Conclusions and Future Research

Typical *C. albicans* strains were discovered in both the Plankenburg and Diep Rivers, both previously reported as polluted wetland habitats (Paulse *et al.*, 2009). However, the presence of *C. albicans* was zone-specific, only evident in rock-filtered and plant-filtered zones early in the rainy season and late in the dry season. As the rainy season progressed, and total yeast and coliform numbers in all the zones dropped; *C. albicans* was no longer evident in rock-filtered zones in the wetlands. Nevertheless, *C. albicans* numbers remained constant in plant debris-rich zones. No *C. albicans* was found in clear, flowing river water during this study.

The contribution of potential carbon sources in this environment to the survival of *C. albicans* was investigated, and saprophytic growth on decaying plant matter was demonstrated, rather than commensal survival in the rhizosphere of wetland flora. Under anaerobic conditions, wetland plant debris was shown to increase the competitive survival of *C. albicans* and wetland plant debris nutrient profiles showed sufficient carbon and nitrogen sources for yeast growth under anaerobic conditions. Indications from this study are that cellulose and fibre degradation play no role in the survival of *C. albicans* in this wetland environment, and carbon is primarily sourced from simple monosaccharides released during the natural degradation process of lignocellulose.

These are substantial indications that the oxygen-limited, reducing, plant debris-rich zones of wetlands and river banks - so similar to the natural gastrointestinal reservoir of sewage-borne *C. albicans* - may harbour the environmental niche of *C. albicans*. Further elucidation of its ability to survive could involve investigating the potential of *C. albicans* to form biofilms on decomposing plant matter in this environment. This may improve its competitive ability, since microbiota competition has been suggested in this work as a limiting factor in the survival of this yeast in a wetland habitat. Also, the demonstration of the conjugation of *C. albicans* on wetland plant debris would further support the postulation of De Hoog *et al*. (2000), that both the assimilative thallus and reproductive structures are indicative of a niche.

The potential niche discussed in this work poses a threat to an HIV/AIDS population that habitually frequents such regions for necessary water-related tasks such as bathing and washing. A more comprehensive survey is required within both polluted and pristine wetland and river bank habitats, as well as long term survival studies of *C. albicans* within these aquatic habitats. Information obtained in

such studies is potentially useful to inform immunocompromised populations of the risks associated with these oxygen-limited, reducing zones; confirming the safety of flowing versus stagnant water, particularly in terms of pathogen survival. Wetlands employed specifically for sewage treatment should also be surveyed for *C. albicans* growth, since the indications obtained in the present study are that a wetland system utilised for pathogen removal may provide a niche for this opportunistic pathogen to survive and even thrive.

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Appendix A

Sequence confirmation of randomly selected *Candida albicans* strains isolated from the Plankenburg River. A, B and C represent randomly selected Plankenburg river isolate sequences, amplified from the ITS gene region. Isolates were chosen based on preliminary Candiselect4 identification. F represents the nearest related FASTA sequence downloaded from Genbank **(Query coverage; Maximum Identity)**.

A. >B08_Calb5_F63___062.ab1

TATGCCTCAGTAGCGGCGAGTGAGCGGCAAAAGCTCAAATTTGAAATCTGGCGTCTTTGGCGTCCGAGT
TGTAATTTGAAGAAGGTATCTTTGGGCCCGGCTCTTGTCTATGTTCCTTGGAACAGGACGTCACAGAGG
GTGAGAATCCCGTGCGATGAGATGACCCGGGTCTGTGTAAAGTTCCTTCGACGAGTCGAGTTGTTTGGG
AATGCAGCTCTAAGTGGGTGGTAAATTCCATCTAAAGCTAAATATTGGCGAGAGACCGATAGCGAACAA
GTACAGTGATGGAAAGATGAAAAGAACTTTGAAAAGAGAGTGAAAAAGTACGTGAAATTGTTGAAAGGG
AAGGGCTTGAGATCAGACTTGGTATTTTGCATGCTGCTCTCTCGGGGGCGGCCGCTGCGGTTTACCGGG
CCAGCATCGGTTTGGAGCGGCAGGATAATGGCGGAGGAATGTGGCACGGCTTCTGCTGTGTGTTATAGC
CTCTGACGATACTGCCAGCCTAGACCGAGGACTGCGGTTTTTACCTAGGATGTTGGCATAATGATCTTA
AGTCGCCCGTCTTG

B. >C08_Calb6_F63___060.ab1

AGTAGCGGCGAGTGAAGCGGCAAAAGCTCAAATTTGAAATCTGGCGTCTTTGGCGTCCGAGTTGTAATT
TGAAGAAGGTATCTTTGGGCCCGGCTCTTGTCTATGTTCCTTGGAACAGGACGTCACAGAGGGTGAGAA
TCCCGTGCGATGAGATGACCCGGGTCTGTGTAAAGTTCCTTCGACGAGTCGAGTTGTTTGGGAATGCAG
CTCTAAGTGGGTGGTAAATTCCATCTAAAGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACAGT
GATGGAAAGATGAAAAGAACTTTGAAAAGAGAGTGAAAAAGTACGTGAAATTGTTGAAAGGGAAGGGCT
TGAGATCAGACTTGGTATTTTGCATGCTGCTCTCTCGGGGGCGGCCGCTGCGGTTTACCGGGCCAGCAT
CGGTTTGGAGCGGCAGGATAATGGCGGAGGAATGTGGCACGGCTTCTGCTGTGTGTTATAGCCTCTGAC
GATACTGCCAGCCTAGACCGAGGACTGCGGTTTTTACCTAGGATGTTGGCATAATGATCT

C. >H07_Calb3_F63___049.ab1

TGCATATCAATAAGCGGAGGAAAAGAAACCAACAGGGATTGCCTCAGTAGCGGCGAGTGAAGCGGCAAA
AGCTCAAATTTGAAATCTGGCGTCTTTGGCGTCCGAGTTGTAATTTGAAGAAGGTATCTTTGGGCCCGG
CTCTTGTCTATGTTCCTTGGAACAGGACGTCACAGAGGGTGAGAATCCCGTGCGATGAGATGACCCGGG
TCTGTGTAAAGTTCCTTCGACGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTAAATTCCAT

CTAAAGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACAGTGATGGAAAGATGAAAAGAACTTTG
AAAAGAGAGTGAAAAAGTACGTGAAATTGTTGAAAGGGAAGGGCTTGAGATCAGACTTGGTATTTTGCA
TGCTGCTCTCTCGGGGGCGGCCGCTGCGGTTTACCGGGCCAGCATCGGTTTGGAGCGGCAGGATAATGG
CGGAGGAATGTGGCACGGCTTCTGCTGTGTGTTATAGCCTCTGACGATACTGCCAGCCTAGACCGAGGA
CTGCGGTTTTTACCTAGGATGTTGGCATAATGATCTTAAGTCGCCCGTCTTGAAACACGG

F. (A & B) >gi|226426539|gb|FJ627956.1| *Candida albicans* isolate FSMP-Y7 26S ribosomal RNA gene, partial sequence
GCATATCAATAAGCGGAGGAAAAGAAACCAACAGGGATTGCCTCAGTAGCGGCGAGTGAAGCGGCAAAA
GCTCAAATTTGAAATCTGGCGTCTTTGGCGTCCGAGTTGTAATTTGAAGAAGGTATCTTTGGGCCCGGC
TCTTGTCTATGTTCCTTGGAACAGGACGTCACAGAGGGTGAGAATCCCGTGCGATGAGATGACCCGGGT
CTGTGTAAAGTTCCTTCGACGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTAAATTCCATC
TAAAGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACAGTGATGGAAAGATGAAAAGAACTTTGA
AAAGAGAGTGAAAAAGTACGTGAAATTGTTGAAAGGGAAGGGCTTGAGATCAGACTTGGTATTTTGCAT
GCTGCTCTCTCGGGGGCGGCCGCTGCGGTTTACCGGGCCAGCATCGGTTTGGAGCGGCAGGATAATGGC
GGAGGAATGTGGCACGGCTTCTGCTGTGTGTTATAGCCTCTGACGATACTGCCAGCCTAGACCGAGGAC
TGCGGTTTTTACCTAGGATGTTGGCATAATGATCTTAAGTCGCCCGTCTTGAACACGGACC **(100 %; 100 %)**

F. (C) >gi|5732686|gb|AF156537.1|AF156537 *Candida albicans* strain TA62 26S ribosomal RNA gene, partial sequence
TGCATATCAATAAGCGGAGGAAAAGAAACCAACAGGGATTGCCTCAGTAGCGGCGAGTGAAGCGGCAAA
AGCTCAAATTTGAAATCTGGCGTCTTTGGCGTCCGAGTTGTAATTTGAAGAAGGTATCTTTGGGCCCGG
CTCTTGTCTATGTTCCTTGGAACAGGACGTCACAGAGGGTGAGAATCCCGTGCGATGAGATGACCCGGG
TCTGTGTAAAGTTCCTTCGACGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTAAATTCCAT
CTAAAGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACAGTGATGGAAAGATGAAAAGAACTTTG
AAAAGAGAGTGAAAAAGTACGTGAAATTGTTGAAAGGGAAGGGCTTGAGATCAGACTTGGTATTTTGCA
TGCTGCTCTCTCGGGGGCGGCCGCTGCGGTTTACCGGGCCAGCATCGGTTTGGAGCGGCAGGATAATGG
CGGAGGAATGTGGCACGGCTTCTGCTGTGTGTTATAGCCTCTGACGATACTGCCAGCCTAGACCGAGGA
CTGCGGTTTTTAACCTAGGATGTTGGCATAATGATCTTAAGTCGCCCGTCTTGAAACACGG **(100 %; 99 %)**

Appendix B

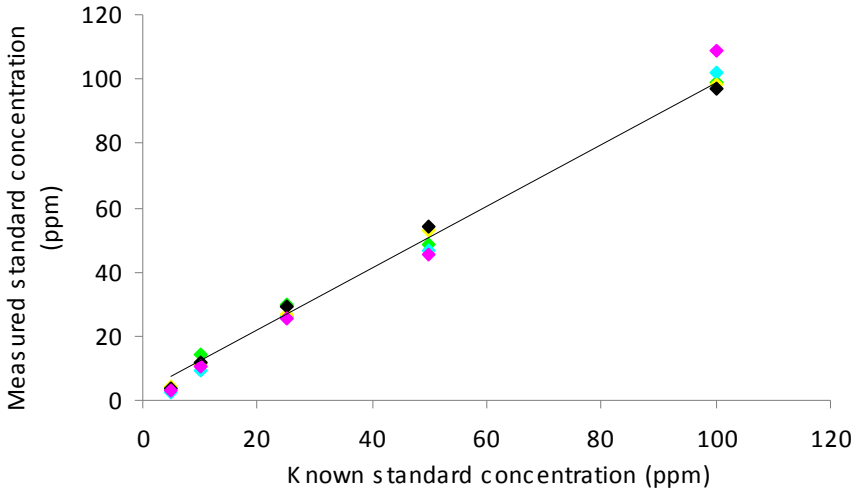

Figure 1. Standard curves for the five mono- and disaccharides analysed with HPLC. The standards for individual carbohydrates are depicted by the following colours: arabinose – black; fructose – blue; glucose – green; sucrose – yellow; xylose – pink.

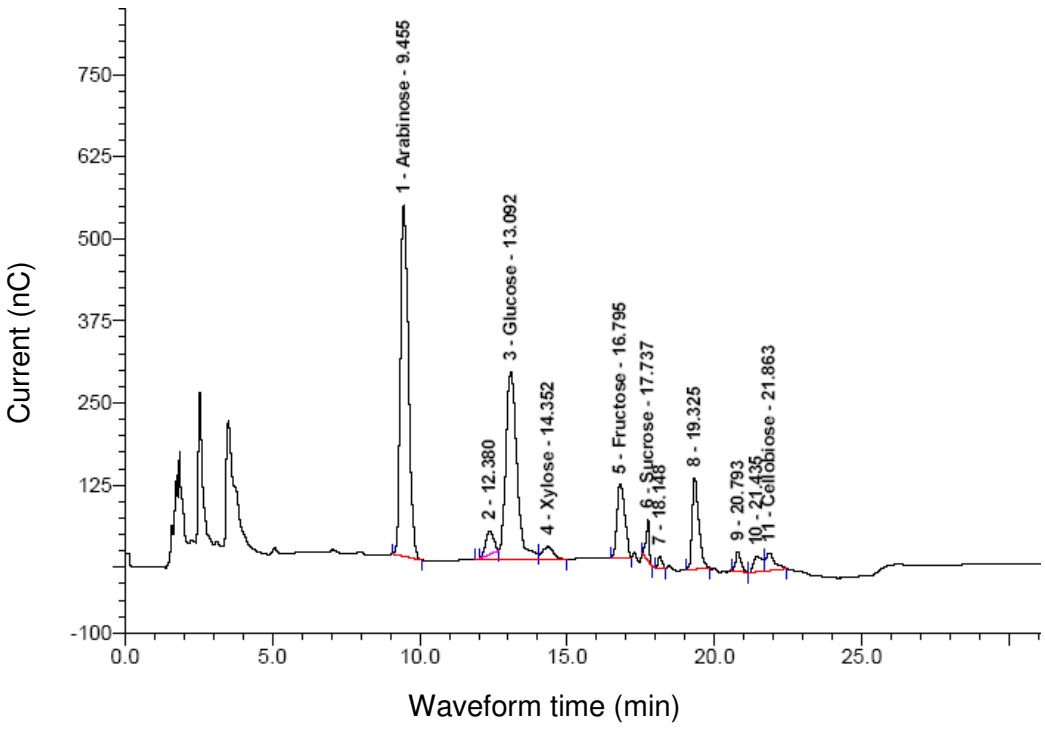

Figure 2a. An integrated I-t (current versus waveform time) plot of the 3D amperometry data generated by HPLC for the uninoculated wetland plant debris extract after *ca.* 7 h anaerobic incubation at 30 ℃. Peaks for each mono- and disaccharide are measured in nanoCoulombs at respective waveform times (min). The area under each peak represents the amount of each sugar present in the extract.

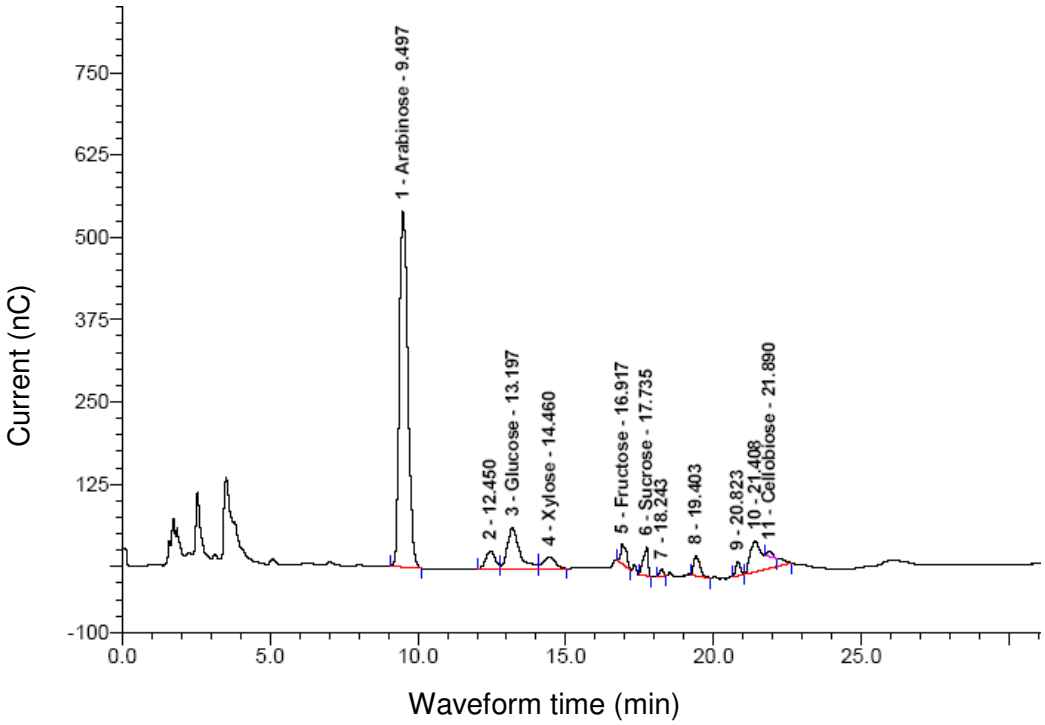

Figure 2b. An integrated I-t (current versus waveform time) plot of the 3D amperometry data generated by HPLC for the wetland plant debris extract after inoculation with *C. albicans* CAB 628-1 and *ca*. 7 h anaerobic incubation at 30 ℃. Peaks for each mono- and disaccharide are measured in nanoCoulombs at respective waveform times (min). The area under each peak represents the amount of each sugar present in the extract. A decrease in peak size, in comparison to Fig 2a, represents sugar assimilation by *C. albicans*.

Appendix C

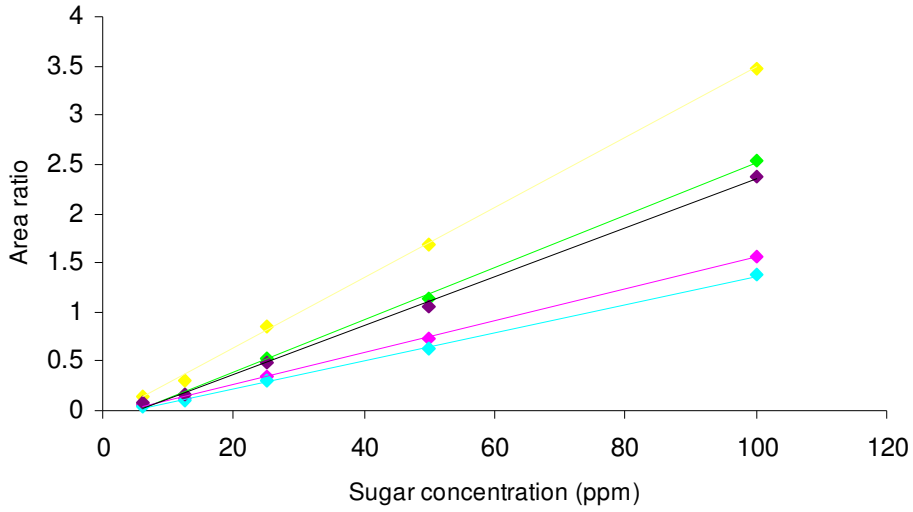

Figure 1. Standard curves for the five mono- and disaccharides analysed with GC-MS. The y axis represents the peak area of each sugar, normalised with the peak area of ribitol, the internal standard. The standards for individual carbohydrates are depicted by the following colours: fructose – blue; galactose – black; glucose – green; sucrose – yellow; xylose – pink.

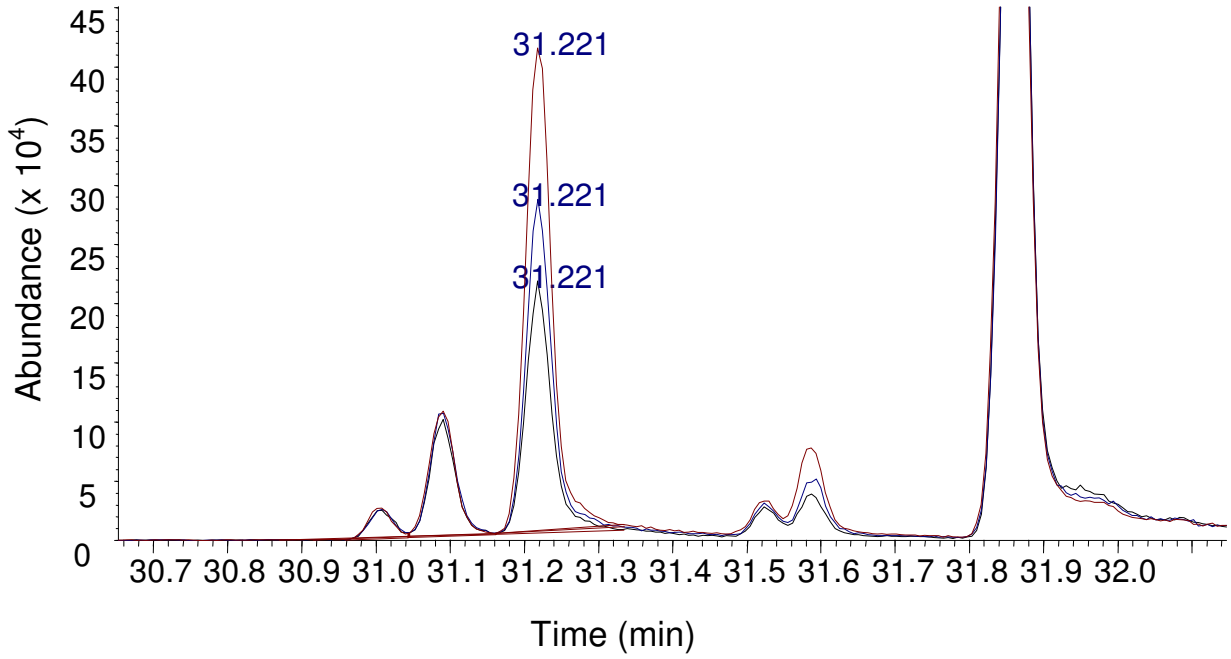

Figure 2. Glucose abundance in one replicate of plant extract after anaerobic culturing with *C. albicans* for 7 h at 30 ℃, depicted by the peak at 31.221 min and measured using GC-MS. The red peak represents the uninoculated plant extract control, the blue peak represents plant extract cultured with *C. albicans* TH 8908 and the black peak represents plant extract cultured with *C. albicans* CAB 628-1.

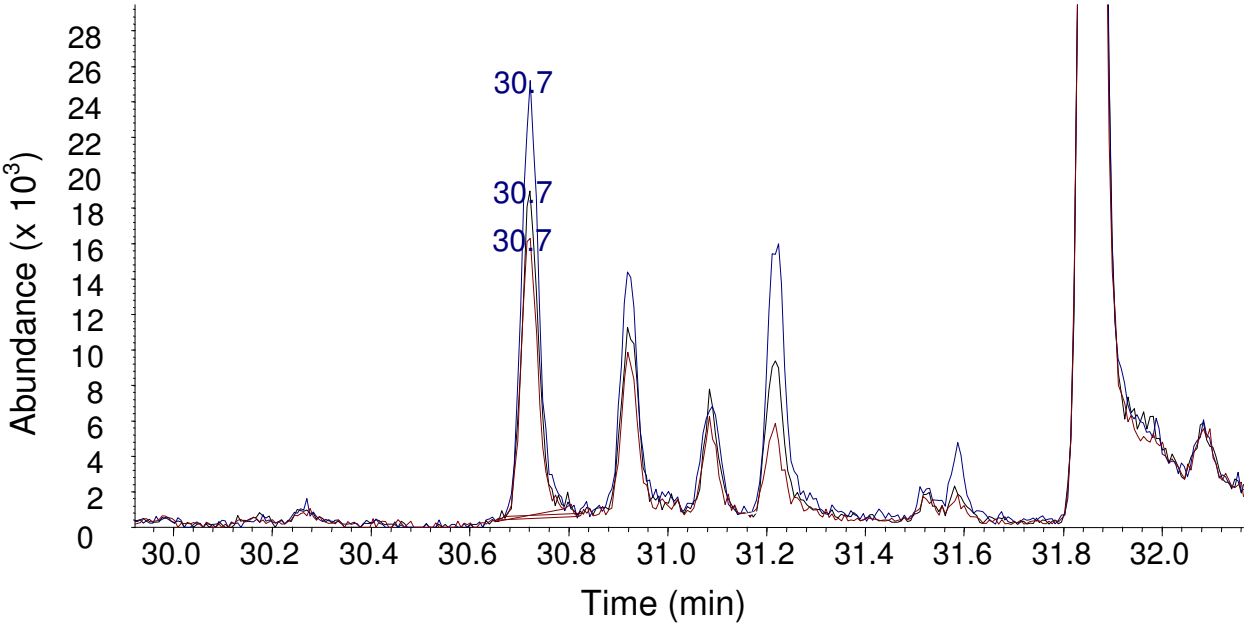

Figure 3. Fructose abundance in one replicate of plant extract after anaerobic culturing with *C. albicans* for 7 h at 30 ℃, depicted by the peak at 30.72 min and measured using GC-MS. The blue peak represents the uninoculated plant extract control, the black peak represents plant extract cultured with *C. albicans* CAB 628-2 and the red peak represents plant extract cultured with *C. albicans* TH 8912.

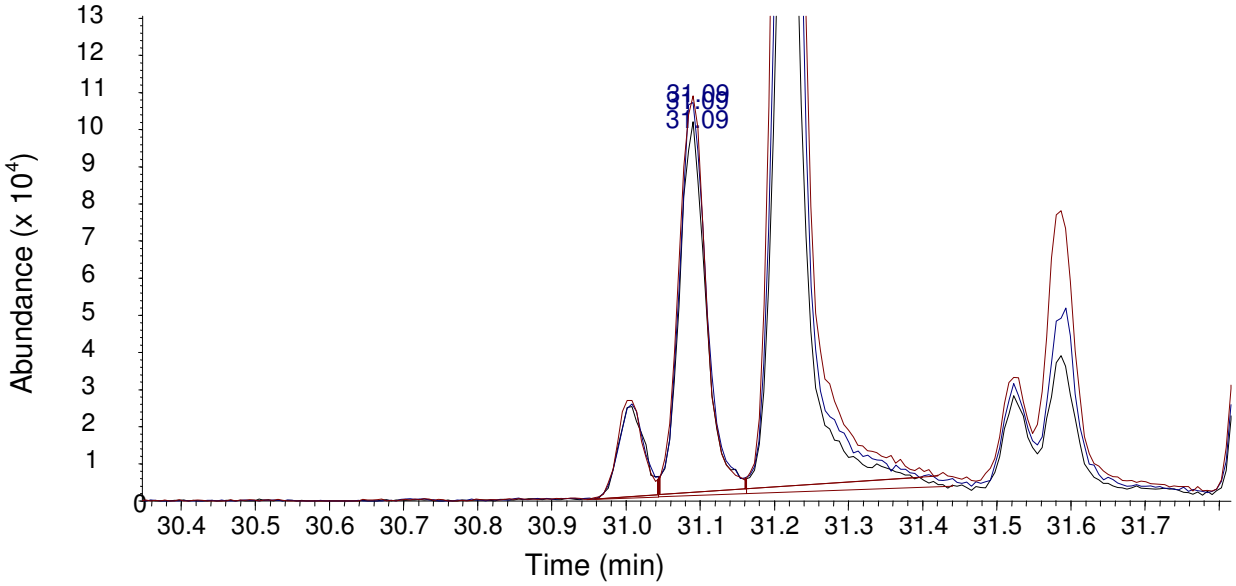

Figure 4. Galactose abundance in one replicate of plant extract after anaerobic culturing with *C. albicans* for 7 h at 30 ℃, depicted by the peak at 31.09 min and measured using GC-MS. The red peak represents the uninoculated plant extract control, the black peak represents plant extract cultured with *C. albicans* CAB 628-2 and the blue peak represents plant extract cultured with *C. albicans* TH 8912.

Appendix D

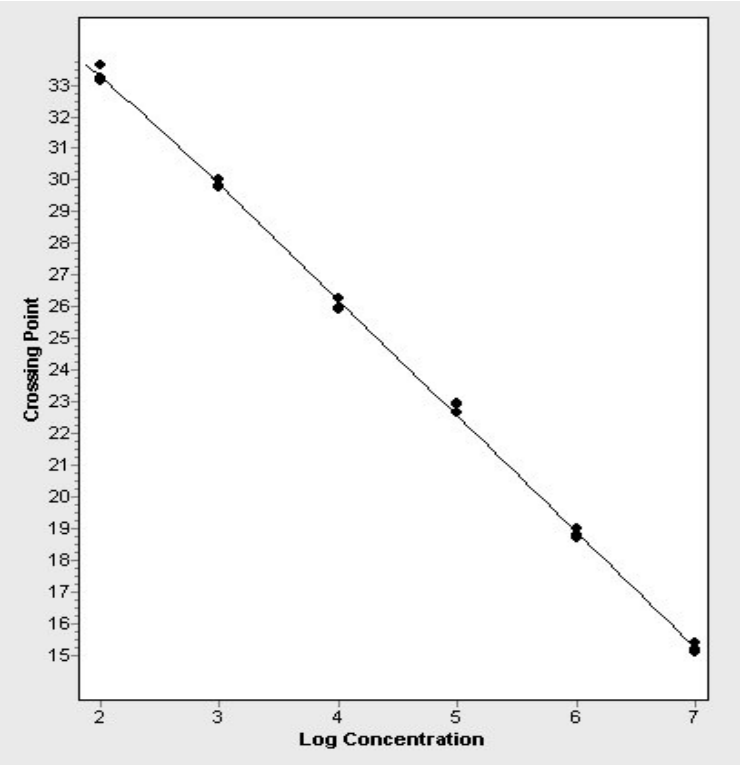

Figure 1. A standard curve representing the crossing points of a concentration range (10^2-10^7 cells/mL) of the target organism (*C. albicans* CAB 628-1) analysed by qRT-PCR. Error = 0.0196; Efficiency = 1.879 (both calculated using Roche LightCycler Software, Version 4.05). The crossing point of unknown samples was used to determine *C. albicans* cell concentrations in comparison to this graph, normalising with both a reference organism (*G. candidum* CBS 109.12) and a calibrator sample of known *C. albicans* CAB 628-1 concentration.